Infection at the Wildlife-livestock-human interface: three systems

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Abstract

Zoonoses involve interactions between at least three species: the pathogen and two hosts, one of which is human and the other a non-human (vertebrate) animal. More than 60% of human infectious diseases are zoonotic, and many have a wildlife host. Urbanisation and human population growth have increased the demand for food and land resources, which have increased interaction between humans, domestic animals and wildlife and thus the potential for cross-species transmission of infections. Most studies of such systems take place in tropical and developing countries where population change and biodiversity makes the emergence of high profile infections (eg Ebola and SARS) more likely. This study, however, focuses on four well known infections within the UK: bovine tuberculosis, water-borne cryptosporidiosis and giardiasis, and campylobacteriosis. The aim of this study was to **investigate, using four infectious diseases of economic and public health importance in the UK as study systems, the role of wildlife in the epidemiology of multihost, zoonotic infections**.

Bovine tuberculosis (bTB) is an important zoonosis in many parts of the world, but human infection is rare in the UK owing to a policy of 'test and cull' in cattle and pasteurisation of milk. However, there has been an epidemic of bTB in British cattle in recent decades, the control of which is complicated by infection in badgers (*Meles meles*) and controversy over the control of wildlife infection. I investigated TB in badgers in the Cheshire area, located on the edge of the epidemic in England, in collaboration with various stakeholders. Using a road-kill survey, I found *M. bovis* in 20 of 94 badgers: the estimated prevalence of 21.3% (95% CI 14.2-30.6) is comparable to the county-level prevalence found at the core of the epidemic. That all isolates were spoligotype SP25, suggests this is an expansion of infection from neighbouring counties. The directionality of any cross-species transmission of bTB between wildlife and livestock cannot be ascertained from this project. However, it showed that using road-killed badgers is a valuable approach to sampling, especially if combined with the engagement of stakeholders

Cryptosporidium spp and *Giardia duodenalis* are protozoa that can cause diarrhoea in many mammals including humans. Llyn Cowlyd, a major water reservoir in North Wales, had seen annual summer peaks of uncharacterised *Cryptosporidium* spp cysts, without human disease. My study aimed to determine the source(s) of the contamination and to investigate *G. duodenalis* in the same system. Water samples were collected from the reservoir and feeder streams, and faecal samples from livestock and wild rodents. In total, 97 rodents were sampled: 35 (35.7% CI 95% 26.9-45.6%) were positive for *Cryptosporidium spp*. and 11 (11.2% CI 95% 6.4-19%) for *Giardia spp*. Of cryptosporidia detected, 55% were novel genotypes and only 5% *C. parvum (*zoonotic). Of 11 livestock samples, only two samples were positive for *C. parvum* and *G. duodenalis*. All the rodent *Giardia* belonged to an apparently novel assemblage while livestock *Giardia* belonged to non-zoonotic assemblage E. The water samples contained *C. ubiquitum*, *C. parvum*, and *G. duodenalis* assemblages E, A (zoonotic), and the novel rodent assemblage.

Campylobacter jejuni and *C. coli*, are common causes of diarrhoeal disease in humans. Infection is common in a wide range of livestock and wildlife species, usually, however, without disease. The aim of this study was to investigate the potential role of wild birds in the epidemiology of campylobacteriosis on dairy and poultry units already studied in depth as part of a larger research programme. In total, 299 birds were sampled and 14 (4.7% CI 95% 2.8-7.7%) were positive for *C. jejuni*. Multi-locus sequence typing showed each isolate to be different, and many of these sequence types found in wild birds have not been associated with human disease.

Overall, these results show that while at first sight wildlife might be assumed to be potential sources of zoonotic infections, further characterisation of the agents involved often revealed separate cycles of infection in wildlife, livestock and humans.

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Declaration of authorship

I Elsa Sandoval Barron declare that this thesis and all the work presented in it is my own, I have acknowledged all the main sources of help and all the specific sources of information.

I also declare that the studies presented in this thesis were all approved by the appropriate ethics panels at the University of Liverpool and the University of Nottingham.

Signed……..

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1. Introduction

Knowledge of the importance of interdisciplinary approaches to deal with disease has been around for hundreds of years; indeed, many ancient cultures preached the relevance of respecting nature and caring for the health of animals and plants, as well as the abiotic environment, in order for humans to thrive. Notes on this importance can be found again and again throughout records of human history, from Hippocrates (460 BCE – 367 BCE) and Aristotle (384 BCE – 322 BCE), to Bourgelat (1712-1779) and Virchow (1821-1902) (Evans & Leighton, 2014). However, it was not until the late 20th century that Steele and Shwabe called for a more integrated approach between animal, human and environmental health. In his book *Veterinary Medicine and Human Health,* Schwabe *(1969)*, referred to the concept of "one medicine" and expressed the view that 'the critical needs of man include the combating of diseases, ensuring enough food, adequate environmental quality and a society in which humane values prevail'.

Soon the concept of 'one medicine' would become insufficient to portray the interactions of human and animal health, let alone include ecosystem, wildlife and society aspects into the concept; therefore a new approach named 'one health' was born (Zinsstag et al. 2005). According to the 'One Health Initiative', this concept seeks to "promote, improve and defend the health and wellbeing of all species by enhancing cooperation and collaboration between physicians, veterinarians, other scientific health and environment professionals" (http://www.onehealthinitiative.com). However, it has been argued that this new approach was not enough to encompass the in-depth understanding of ecological systems, where direct animal-human health is just a part of a much bigger picture. Hence, the development of the idea of 'ecosystems approach to health' or eco-health', which intends to explore the relationship between different components of an ecosystem in order to determine priority determinants to human health and at the same time, evaluate the sustainability of that ecosystem based on management measures rather than conventional health interventions (Forget and Lebel 2001).

Changes in ecosystems due to human or natural interventions can have an impact on human and animal health; for example, an improvement in housing conditions in an area of Paraguay helped reduced the contact between humans and *Triatomas*; (the vector of *Trypanosoma cruzi*, causal agent of Chagas disease) reducing the risk of contracting the disease (Rojas de Arias et al. 1999). Another example of these interactions is brucellosis, which in humans is linked to brucellosis dynamics in sheep and cattle populations, which are in turn, determined by ecological factors such as feed availability, husbandry measures and land resources. Therefore, by managing the ecosystem in which livestock live, we can influence the burden of disease in humans (Zinsstag et al. 2011). Hence it is no surprise that political, economic, cultural and social aspects are deeply related with health and well-being of animals and ecosystems and vice versa.

Regardless of the name we give it, the importance of this approach is that it emphasizes the interaction between mainly, but not only, human and veterinary medicine. It also highlights the importance of the involvement of other disciplines that are not directly linked to health sciences, through collaboration networks, in the resolution of human and other animal diseases.

An important part of the studies described in this thesis was the development of networks (some formal, some informal) of participants who would work together collaboratively and that would allow the fulfilment of the objectives of each specific project. These networks consisted of different medical and veterinary experts, as well as others, including local authorities, farmers, academics, epidemiologists, statisticians, wildlife charities and foundations, local animal rescue centres, private utilities companies and reference laboratories.

1.1 Zoonoses

1.1.1 Definitions and importance

The World Health Organisation (WHO) defines zoonoses as "any disease or infection naturally transmissible from vertebrate animals to humans and vice versa" (WHO, 2016c). This transmission can occur indirectly through contaminated food, water, environment or fomites, or through direct contact with infected invertebrate animals or 'vectors'. For a zoonosis to be considered emerging it needs to be a "newly recognised or newly evolved disease, or if it has occurred previously it must show increase in incidence or expansion in a geographic, host or vector range" (WHO, 2016a). This means that in some cases, diseases that are considered endemic can become emerging if they enter new hosts, enter human populations or appear in new geographical areas (Emerging infectious diseases 'EIDs').

The importance of zoonotic and emerging zoonotic diseases is that most human infectious diseases are zoonotic, with almost 61% of them being transmissible from animals directly or through invertebrate 'vectors'. These agents can potentially cause disease in both humans and other animals, have present wildlife 'reservoirs', with or without clinical disease, or have originated from livestock or wildlife but be now adapted to human hosts (Cleaveland et al., 2001, Daszak et al., 2001, Taylor et al., 2001).

A zoonosis involves the interaction of at least three species; one pathogen and two hosts of which one is human. The nature and number of the non-human hosts can make this model a more complex one especially if vectors -insects that transmit pathogens- like arthropods are involved (Karesh et al., 2012).

Zoonotic pathogens may be confined largely to non-human animal reservoirs with occasional transmission to humans and no further onward transmission from humans (rabies might fall into this category), or, like Ebola, might circulate mainly in non-human hosts but be able to cause outbreaks in human populations and possibly even be transmitted to other animals. Other zoonotic agents may be well established in both human and non-human animal populations, with transmission occurring between all hosts, for example bovine tuberculosis or salmonellosis (Slingenbergh et al., 2004).

One of the issues in discussing zoonoses is terminology. As the one thing all zoonoses have in common is a human host, much of the terminology has come from medicine, and sometimes

ecological and evolutionary terms have been misapplied. This means that similar terms can mean different things to different people, which can be of obvious concern in a fundamentally interdisciplinary area. Recently, there have been several publications that discuss and attempt to clarify – or at least pin down – some of these terms, and Table 1.1 summarises the most common terminology proposed for the epidemiological study of zoonotic diseases.

The majority of EIDs are the result of a change in pathogen and/or host ecology. For example, climate change is predicted to have a great impact on vector distribution, migration patterns and pathogen survival outside the host (Morse, 1995, Schrag and Wiener, 1995). Other anthropogenic risk factors might include social inequalities, changes in social behaviour, travel, importation and exportation of goods and war (Farmer, 1996), as well as new developments in human and veterinary medicine and in animal husbandry. Sometimes, one EID can lead to another: changes in availability of the preferred host, particularly in vector mediated transmission, can make feeding patterns of the vector shift from other animals to humans. For example, the introduction of rinderpest in Africa led to a decrease in cattle and wildebeest numbers which made the tse-tse fly more likely to feed on humans, causing in turn an epidemic of sleeping sickness (African trypanosomiasis) (Ford, 1971).

Table 1.1. Proposed terminology for the epidemiological study of zoonoses, with definitions as used in this thesis.

Term	Definition	Reference
Target population	Population of interest of a study, control programme, etc	(Haydon et al., 2002)
Non-target population	Potentially susceptible populations that are directly or indirectly connected epidemiologically to the target population	(Haydon et al., 2002)
Critical community size (CCS)	Minimum size of a population needed for a pathogen to persist indefinitely	(Haydon et al., 2002, Viana et al., 2014)
Maintenance population	Populations larger than the CCS	(Haydon et al., 2002, Viana et al., 2014)
Nonmaintenance population	Populations that naturally or through control measures are smaller than the CCS	(Haydon et al., 2002)
Maintenance community	Transmission of infection between any set of connected hosts, including nonmaintenance hosts, that allows the pathogen to survive	(Haydon et al., 2002, Viana et al., 2014)
Source population	Any population that can potentially transmit a pathogen or an infection to the target population	(Haydon et al., 2002)
Reservoir of infection	Ecologic system in which an infectious agent can survive indefinitely through one or more populations or environments epidemiologically connected and that can transmit the agent to the target population. Reservoirs can be wildlife species, domestic species or subsets of the same species	(Haydon et al., 2002, Viana et al., 2014, Ashford, 2003)
Reservoir host	Vertebrate hosts that are an essential part of the reservoir of infection	(Ashford, 2003)
Reservoir capacity	Potential of a reservoir of infection to support infection indefinitely without external imports	(Viana et al., 2014)
Incidental host	Species or subpopulations hat become infected but are not essential for survival of the pathogen in the maintenance population	(Ashford, 2003)
Liaison host	Incidental hosts capable of transmitting pathogens from reservoir hosts to target populations or other incidental hosts. Amplifying hosts are liaison hosts in which the parasite numbers are increased so as to make infection of the target host possible	(Harcourt, 2011)
Ring-fencing	Isolation of the target population when its size is smaller than the CCS and thus leading to the pathogen extinction	(Haydon et al., 2002)
Spill-over	Transmission of infectious agents from one population to another – eg spill over from a maintenance host population to sympatric populations in which the infection does not usually occur	(Daszak et al., 2000)
Spill-over host	Animals susceptible to the pathogen in question but when the original source of infection is removed, prevalence declines and infection is no longer sustained within the spill-over population	(Cousins, 2001)
Spill-back	Also called "reverse spill-over"	(Daszak et al., 2000)
Biological pollution	Anthropogenic introduction of biota to new habitats. This can be accidentally or intentionally	(Horan et al., 2002)
Pathogen pollution	Anthropogenic introduction of pathogens to new habitats	(Aguirre and Tabor, 2008, Daszak et al., 2000)
Reverse zoonosis	Pathogens with human reservoirs that can be transmitted to other vertebrates	(Messenger et al., 2014)

1.1.2 The wildlife-livestock-human interface

The areas where production animals, wildlife species and humans converge is sometimes referred to as the wildlife-livestock-human interface. It includes a range of farming systems, wildlife habitats and human expansion areas and can be classified according to disease transmission or agricultural use; in both cases, these interfaces are dynamic; bidirectional; multidimensional and most commonly, located at the physical contact point where all three populations access communal resources such as water, food or rangeland. However, for classification according to disease transmission location and time period of disease transmission between wildlife and non-wildlife species are the two most critical factors (Siembieda et al. 2011) and contact can be direct through contaminated aerosols, feed, water or environment or indirect through flightless, winged and mechanical vectors (Bengis et al. 2002).

Examples of the interactions and diseases at these interfaces include domestic species introduced to naïve areas where some of their pathogens found new susceptible wildlife hosts with, in some cases, devastating consequences, such as canine distemper introduced through domestic dogs into the Serengeti ecosystem causing the death of 30% of the lion population and the major decline of the wild dog through direct contact (Alexander and Appel, 1994, Roelke-Parker et al. 1996). Rabies is a clear example of bidirectional transmission through direct contact between wildlife and domestic species with spill over to humans; in some areas the majority of cases are reported in domestic dog and cattle, however, in Africa and North America various wildlife species are considered important contributors to the spread and maintenance of the disease (Bengis et al. 2002).

Examples of transmission through indirect contact include vector-borne diseases such as West Nile virus transmitted through mosquitoes from wild birds to various domestic species and man (Campbell et al. 2002) and Lyme disease transmitted to domestic species and humans through the *Ixodes* tick from small mammals and white tail deer (Wood and Lafferty, 2013). Some diseases like tularemia can be transmitted through direct contact with wildlife such as voles and rabbits or indirectly through mosquitoes and ticks (Eliasson et al. 2002).

As mentioned above, different farming systems lead to different wildlife-livestock interactions, either directly or indirectly. For example, in many intensive farming systems where animals are confined indoors, the interaction between wildlife species and livestock is mostly limited to a small range of small mammals like woodmice, voles and hedgehogs and birds such as sparrows and pigeons, although indirect contact with larger species for example, wild deer or wild boars, can occur

through manure, waste and aerosols – perhaps largely from livestock to wildlife. On the other hand, free range or extensive farming permits livestock to roam more freely outdoors allowing, at least in theory, more contact with a wider range of species of wildlife (Navarro-Gonzalez et al., 2016). This interface is commonly classified depending on agricultural intensification and environmental change, for example, a 'pristine' ecosystem would consist of no livestock but, perhaps some human incursion for harvesting wildlife and other resources and high biodiversity. In contrast, a 'managed landscape, typical of most of the UK, would be an area of intensive farming, low biodiversity, many livestock and contact with only certain types of wildlife, mainly adaptable 'peridomestic' species. 'Ecotones' and 'evolving landscape' would be the intermediate classifications between these two extremes and are explained in more detail in Table 1.2 (Jones et al., 2013). These classifications and the EIDs linked to them are summarised in Table 1.2.

It is important not to overlook other domestic animals at the livestock-wildlife-human interface; for example pets can be reservoirs of infection, incidental hosts, liaison hosts or even source populations of zoonotic diseases for humans and other animals. Companion and pet animals have been sources of a range of human diseases including rabies (e.g. dogs), leptospirosis (e.g. dogs and rats), tuberculosis (e.g. cats), toxoplasmosis (cats), brucellosis (dogs), and hantavirus (pet rats). In some areas, such as urban settings, the abundance of companion animals is greater than food-production animals. As an example, 46% and 63% of households in the UK and the USA respectively, own at least one pet, and in Europe there are approximately 70 million pet-owning households (PFMA, 2015a, PFMA, 2015b).

In some environments, such as urban or peri-urban areas, the population dynamics and natural behaviours of wildlife species have changed, for example, to make use of food or shelter resources. This is particularly true for highly adaptable wildlife species, e.g. rats, house mice, foxes and house sparrows. Indeed the very notion of 'farmland birds', and the names of some now common species (house sparrow, barn swallow, field vole, etc.) demonstrate how long term this adaptation to anthropogenic change has existed in a country such as the UK. Attraction to human settlement, for food or shelter, can lead to population densities of such species being considerable higher near human settlements than in more pristine landscapes, potentially posing an increased threat for pathogen transmission as contact between wildlife, humans, and domestic animals becomes more frequent (Bradley and Altizer, 2007, Mackenstedt et al., 2015, Soulsbury and White, 2015).

Table 1.2. Classification of the wildlife-livestock-human interface according to agricultural use and biodiversity and EIDs associated with each area (Jones et al., 2013).

Classification	Agricultural use	Biodiversity and Contact with wildlife	Examples of EIDs associated with these areas
Pristine	No livestock, no farming practices	High biodiversity and native wildlife species	Ebola, HIV, SARS
Ecotones	Farming edges, transition areas. Few livestock	High but decreasing biodiversity. Some native and some adaptable wildlife species	Bat rabies, Nipah virus
Evolving landscape	Intensive "backyard" farming. Increasing livestock	Low biodiversity, increasing populations of adaptable (peridomestic) species	Avian influenza
Managed landscape	Intensive farming. Many livestock	Few native wildlife species but increased populations of peridomestic ones	West Nile virus, Lyme disease

Urbanization, population increase, new developments in transport methods and changes in agricultural and farming practices have inevitably influenced the interaction between wildlife and humans through increased direct or indirect contact between species (Morse, 1995). The role that wildlife play in zoonotic and emerging zoonotic diseases in humans has been known for some time, with examples of this relationship observed throughout history; for example, the dispersal of bubonic plague in medieval Europe through the inadvertently importation of rats from Asia via the silk trade route (Morelli et al. 2010) or more recently, the re-emergence of vampire bat rabies in the Amazon basin (Da Rosa et al. 2006) or the emergence of viral haemorrhagic fevers in South America due to different arenavirus such as Junin, Machupo, Guanarito and Sabia, responsible for Argentinian, Bolivian, Venezuelan and Brazil haemorrhagic fevers respectively and all linked to increased contact with different sylvatic rodents (Charrel and Lamballerie, 2003).

One indirect role of wildlife on the appearance of EIDs in human populations is through the sourcing, handling and consumption of bushmeat, for example, some simian foamy virus and EBOLA outbreaks have been linked to contact and consumption of non-human primates (Georges-Courbot et al. 1997, Chomel et al. 2007).

On the other hand, EIDs affecting wildlife have had devastating effects in populations, for example, the rinderpest panzootic in Africa –named the Great African Pandemic- at the end of the 19th century, which caused the death of more than 90% of buffalos and other susceptible wild species (Scott, 1964. Plowright, 1982).

However, unlike EIDs affecting production animals, interest in wildlife EIDs is relatively recent, and with great focus in endangered species (Daszak et al., 2000). The introduction of alien species, through biological or pathogen pollution, has become the leading cause of extinction of wildlife species after habitat loss (Pimm et al., 1995, Vitousek, 1997). Knock-on effects, which involve secondary species altered by declines in directly affected populations have also been demonstrated (Daszak et al., 2000).

The introduction of pathogens, or spill over, to wildlife populations through the expansion of domestic animal and human populations can eventually lead to spill back (reverse zoonosis). Unfortunately, it is often not until this point that most people become aware of diseases affecting wildlife and control becomes politically charged as conservation and commercial interests clash (Daszak et al., 2000, Daszak et al., 2001).

1.1.3 Zoonoses and public health

The risk to public health posed by zoonotic diseases is a big driver for legislative, political and economic changes, as well as research. However, as resources are limited, zoonoses are usually prioritised, often using criteria such as: the number and type of risk factors, transmission speed, human-to-human transmission, case fatality rate, availability of treatment or preventive treatment (vaccination) and cost of the disease *vs* cost of control (Van der Giessen et al., 2004).

Worldwide, zoonotic diseases have an important economic impact. Over the last ten years, it has been estimated that direct costs were around $20 billion, with an additional $200 billion of indirect losses. However, calculations of the costs of zoonotic diseases are most likely underestimated, as house-hold livelihood and patient-based private costs are often unavailable (Narrod et al., 2012).

Direct expenses due to zoonoses are mostly health-care related. Nevertheless, losses in farming, food-production and retail industry and even tourism can have major impacts in the economy. For example, controlling Lyme disease in the USA costs approximately $45 million per case (Maes et al., 1998). In 1994, the cost of prophylaxis of 665 people after exposure to one rabid kitten in a pet store in New Hampshire was close to $1.1 million (Daszak et al., 2000). In 1998 the pig farming industry in Malaysia suffer a loss of nearly 60% of pig farms and more than $120 million in exports after an outbreak of Nipah virus (Daszak et al., 2001). The disruption in commerce due to outbreaks of SARS during 2003 has been calculated as having cost the world economy an estimated $50 billion, despite causing illness in only 9,000 people and less than 800 deaths worldwide (Knobler et al., 2004, Smith, 2006). Costs other than economic have also been observed. For example, in 1999 the Food and Drug Administration in the USA (FDA) banned blood donations from people who had spent more than six months in Britain between 1980 and 1997 due to the BSE epidemic, costing the American Red Cross an estimated loss of 2.2% in donations (Gottlieb, 1999).

Table 1.3 shows zoonoses found in the European Union, categorised according to their primary reservoir and/or mode of transmission, as well as whether they are endemic or newly introduced.
More formally, within the European Union, the list of communicable (contagious) diseases that are considered zoonotic and notifiable are shown in Table 1.4, some of which have obligatory monitoring programmes across the EU.

In the UK, monitoring diseases in animals is viewed as important in order to protect animal welfare, the agricultural industry, wildlife populations and, of course, the human population. Animal diseases

that are notifiable are shown in table 1.5 (APHA, 2015a) – the main criteria for inclusions, however, are to do with economic cost and ability to control rather than necessarily zoonotic potential.

Table 1.3. Zoonoses characterization in Europe and some examples according to their status as endemic with potential to emerge or newly introduced to the EU (Van der Giessen et al., 2004, Civen and Ngo, 2008, Butler, 2015, Giangaspero, 2013, WHO, 2012b, WHO, 2016b).

Category	Situation in EU	Zoonosis
Vector-borne diseases (mosquito, flea, ticks)	Endemic with potential to emerge and occasional outbreaks	West Nile Virus, tick-borne encephalitis, Lyme borreliosis, Crimean-Congo haemorrhagic fever (Nairo virus), tick-borne Rickettsiae, Arbia,Corfu, Naples, Radi, Sicilian and Toscan virus, Leishmaniasis, plague, murine typhus (*Rickettsia typhi*), cat scratch disease (*Bartonella herselae*)
	Newly introduced zoonoses	Dengue, yellow fever, Rift Valley fever, Trypanosomiasis
Transmitted by direct contact with wildlife	Endemic with potential to emerge	Avian influenza, Hantavirus infection, rabies (classical and European bat Lyssa virus), pox viral infections, tularemia (*Fransiscella tularensis*), leptospirosis, *larva migrans* syndrome (*Baylisacaris spp*.) echinococcosis
	Newly introduced zoonoses	Ebola, Marburg, Lassa , Nipah, Menangle and Hendra viruses
Transmitted by direct contact with production animals and food	Endemic with potential to emerge	Tuberculosis (*Mycobacterium bovis*), brucellosis, salmonellosis, verocytoxin-producing *Escherichia coli*, trichinellosis, toxoplasmosis, hepatitis E, cryptosporidiosis, cysticercosis (*Taenia solium*)
Transmitted by pets		Toxoplasmosis, salmonellosis, campylobacteriosis, Hantavirus, Rabies, bite-related meningitis (*Capnocytophagia carnimorsus*), echinococcosis, cat scratch disease (*Bartonella herselae*)

Table 1.4. List of notifiable Zoonoses in the EU (EC, 2003a, EC, 1998, EC, 2007, EC, 2003b, HPSC, 2016). (*)Diseases of obligatory monitoring according to Directive 2003/99/EC (EC, 2003c).

Anthrax	Q-fever
Avian influenza in humans	Rabies
Botulism	Salmonellosis *
Brucellosis *	Severe Acute Respiratory Syndrome (SARS)
Campylobacteriosis *	Shigellosis
Cryptosporidiosis	Toxoplasmosis
Echinococcosis *	Transmissible spongiform encephalitis
Giardiasis	Trichinosis *
E. coli (VTEC) *	Tuberculosis due to *M. bovis* *
Leptospirosis	Tularemia
Listeriosis *	Viral haemorrhagic fevers
Malaria	West Nile Virus infection
Plague	Yersiniosis

Table 1.5 List of notifiable diseases in animals in the UK. (*) are considered potentially zoonotic (APHA, 2015a).

African Horse Sickness	Equine Viral Arteritis
African Swine Fever	Equine Viral Encephalomyelitis
Anthrax*	Foot and Mouth Disease
Aujeszky's Disease	Glanders and Farcy*
Avian Influenza*	Goat Pox
BSE*	Lumpy Skin Disease
Bluetongue	Newcastle Disease*
Brucella abortus*	Paramyxovirus in pigeons
Brucella melitensis*	Peste des Petits Ruminants
Brucella suis*	Rabies*
Classical Swine Fever	Rift Valley Fever
Contagious Agalactia	Rinderpest
Contagious Bovine Pleuro-pneumonia	Scrapie
Contagious Epididymitis	Sheep Pox
Contagious Equine Metritis	Sheep scab
Dourine	Swine Vesicular Disease
Equine Infectious Anaemia*	Teschen Disease
Enzootic Bovine Leukosis	Tuberculosis (Bovine)*
Epizootic Haemorrhagic Virus Disease	Vesicular Stomatitis
Epizootic Lymphangitis	West Nile Virus*

Food and water-borne zoonoses are important globally; considering that it is assumed the average person consumes 1.8 kg of food per day (Kearney, 2010), and ignoring variation in diet and consumption due to location, socio-economic factors, religious and cultural beliefs, etc., this equates to 13 million tonnes of food consumed by the 7 billion people around the world daily. Given this level of exposure, it is very likely that most people will experience at least one episode of food-borne disease during their lifetime. In the USA alone, 9.4 million cases of food-borne disease are estimated to be acquired domestically every year, of which over 2,500 end in death (Scallan et al., 2011), however, some previous studies have estimated the annual disease burden to be 76 million cases with over 5,000 deaths (Mead et al., 1999, Tauxe, 2002). According to the World Health Organisation, 600 million foodborne diseases are reported every year, of which 550 million involve diarrhoeal disease with an estimated 230,000 deaths (WHO, 2015).

According to some studies, in the EU, the most common food-borne zoonotic pathogens are *Salmonella spp., Campylobacter spp., Listeria monocytogenes, Escherichia coli* (VTEC) and Hepatitis E virus. Other group of pathogens of significant importance include *Toxoplasma gondii,* BSE agent, *Clostridium botulinum, Staphilococcus aureus, Cryptosporidium parvum, Mycobacterium bovis, Echinococcus granulosus, Streptococcus* spp*., Echinococcus multilocularis, Yersinia enterocolitica,*

Mycobacterium avium, Fasciola hepatica, and *Giardia intestinalis* (Cardoen et al., 2009, Navarro-Gonzalez et al., 2016).

The studies in this thesis investigate four of these potential food-borne pathogens of zoonotic importance: *Mycobacterium bovis, Campylobacter spp., Cryptosporidium spp.* and *Giardia intestinalis.* Specifically, the aim was to investigate aspects of the role of wildlife in the maintenance and spread of these pathogens into livestock and the human food chain and water supply in the UK. In each case the role of wildlife is not completely clear, and in some cases controversial. Section 1.2 of this chapter will therefore focus on presenting some background to each of these pathogens.

1.2. Infection at the wildlife-livestock-human interface: three systems

1.2.1. Mycobacterium bovis

Mycobacterium bovis belongs to the *M. tuberculosis* complex, a group of closely related mycobacteria capable of infecting humans and other mammals. This complex also includes *M. tuberculosis, M. africanum, M. canetti, M. caprae, M. microti* and *M. pinnipedii* (Bouakaze et al., 2010). It is thought that mycobacteria are old pathogens of animals that emerged approximately 40,000 years ago (Wirth et al., 2008).

Microscopically, *M. bovis* are long, non-motile, acid-fast rods which grow slowly (growth in culture takes longer than seven days). Two characteristics that distinguish this *Mycobacterium* from *M. tuberculosis* are that *M. bovis* is microaerophilic when freshly isolated (*M. tuberculosis* is highly aerobic) and it grows poorly in glycerol-containing media unless supplemented by pyruvate (a characteristic shared also by *M. microti* and *M. africanum*). The distinguishing characteristic of all *Mycobacterium* species is the hydrophobic cell wall, rich in mycolic acids and arabinogalactan, both responsible for the hardiness of mycobacteria (Bhamidi, 2009). Macroscopically, *M. bovis* colonies are dysgonic, raised with both smooth and rough variants and non-chromatogenic (Magee and Ward, 2012).

Pulmonary infection in humans with *M. bovis* is clinically, pathologically and radiologically indistinguishable from infection with *M. tuberculosis* (Pasquali, 2004), which makes determination of causative agent harder, particularly in rural areas. However, the most common source of infection for humans is contaminated raw milk and raw milk products, and oral (rather than respiratory) infection tends to cause cervical lymphadenopathy and abdominal/enteric tuberculosis rather than pulmonary infection (Hardie and Watson, 1992). Human to human transmission of *M. bovis* is considered rare in healthy individuals with a competent immune system, although not uncommon in developing countries (Cosivi et al., 1998, Evans et al., 2007, Etchechoury et al., 2010). Transmission from humans back to animals has also been reported (Fritsche et al., 2004).

In recent decades the global incidence of *M. bovis* infection in people is thought to have risen, probably linked to an increase in HIV/AIDS cases (Cosivi et al., 1998, Golden and Vikram, 2005, Cicero et al., 2009). Further attempts to estimate the incidence of zoonotic tuberculosis have found that although it has a very low incidence in developed countries (<1.4% of tuberculosis cases) it still poses a risk in developing regions. In Africa and Palestine, for example, 2.8% and 6.5% respectively,

of human tuberculosis cases were found to be due to *M. bovis* (Ereqat et al., 2012, Muller et al., 2013).

Cattle and other domestic bovids are considered reservoir hosts: without control measures, infection can be maintained within these populations indefinitely, as has been seen in areas of Africa and other developing countries (Cosivi et al., 1995).

In cattle, the main routes of infection include oral and respiratory pathways. Other routes are very rare, particularly in areas with bTB control measures, and include cutaneous infection, congenital and genital transmission (Biet et al., 2005).

The lesions seen in cattle depend on the transmission route; lungs, thoracic lymph nodes and sometimes retropharyngeal and submandibular lymph nodes are consistent with respiratory infection, and lesions in mesenteric lymph nodes consistent with oral infection. Lesions in other organs such as udders, kidneys and liver are probably due to haematogenous spread from an initial infection in lungs (Neill et al., 1994). Clinical signs in cattle include weakness, loss of appetite, weight loss, fever, enlarged lymph nodes and intermittent cough and respiratory distress (OIE 2015a).

Other domestic animals are also susceptible to infection with *M. bovis*, including camelids, pigs, sheep, goats, horses, dogs and cats. Camelids and pigs used to be frequently infected with *M. bovis* until control measures, including separating camelids from infected cattle and avoiding feeding pigs with milk and offal products of infected animals, reduced the incidence observed in those populations (Cousins, 2001). More recently, however, in south west and west midland regions of England, limited biosecurity in pig and mixed farms has led to an increase in incidence of *M. bovis*-related tuberculosis. As for cattle, it is believed that this increase is more related to wildlife–pig than cattle-pig transmission (Bailey et al., 2013). Infection in sheep, goats and horses is rare and probably a spill-over from infected cattle. Goats, in fact, have their own species of *Mycobacterium* (*M. caprae*). Dogs and particularly cats are also susceptible to *M. bovis*, and are thought to have been the source of a small number of cases in humans.

Various wildlife species are also considered as maintenance hosts for *M. bovis* in different countries: the European badger (*Meles meles*) in Great Britain and Ireland; American white tailed deer (*Odocoileus virginianus*) in N America; wild boar (*Sus scrofa*) in Spain; African buffalo (*Syncerus caffir*) in Southern Africa; and, brushtail possum in New Zealand (*Trichosurus vulpecula*) (Barron et al.,

2013, Miller and Sweeney, 2013, Palmer, 2013, , Hlokwe et al., 2014). Where strategies aimed to control these populations have succeeded, the decrease in livestock incidence, or even the eradication of the disease, has confirmed the idea that some wildlife species are reservoirs of infection and not just incidental hosts. An example of the success of such strategies is the decrease in bTB prevalence in cattle and deer herds in New Zealand observed between 2001 and 2013, which was attributed mainly to effective wildlife pest control; the brushtail possum is exotic to New Zealand and is regarded as an environmental pest species. However, as Hutchings et al. (2013) suggested, besides wildlife population control methods, extensive monitoring and surveillance of the remaining possum populations as well as extensive data of TB surveillance of cattle and deer herds are of the outmost importance if eradication of disease wants to be achieved.

Other wildlife species have been identified as incidental hosts of *M. bovis* and include a wide range of mammals such as gorillas (*Gorilla gorilla gorilla)* and other non-human primates, lynx (*Lynx pardinus)*, rhinoceros (*Dyceros bicornis minor* and *Ceratotherium simum simum)*, elephants (*Loxodonta Africana* and *Elephas maximus)*, meerkats (*Suricata suricatta)*, cheetahs (Acinocyx jubatus) and lions (*Pathera leo)* (Maas et al., 2013).

A basic part of any control strategy should include reliable testing to identify the disease or pathogen before it can spread through that population. Although many efforts have been made to develop a rapid and reliable diagnostic test for bTB, the internationally approved assay for cattle remains a skin hypersensitivity test, which is based on the animal's immune response to intradermal inoculation of tuberculin (antigen derived from mycobacteria). In the comparative skin test used in the UK, Portugal and Ireland, *Mycobacterium avium* complex tuberculin is also inoculated for comparison: this improves the specificity but decreases the sensitivity of the test (Monaghan et al., 1994). In the UK, the gamma Interferon (IFN-g) test is increasingly used alongside the skin test. These circumstances include skin test negative animals in confirmed new TB incidents in areas of low TB incidence, skin test negative animals in herds in high risk areas with persistent infection and suspected fraudulent reactors, among others. This test measures the cellular response to mycobacterial antigens by detecting, in a sandwich ELISA, the release of IFN-g from sensitised blood lymphocytes exposed to mycobacterial antigens (Strain et al., 2011). The downside of both tests is that they rely on the immune response of the animal to previous exposure to the microorganism, which could lead to false negatives under immunosuppressive circumstances or false positives in those animals previously immunised. Test positive animals are culled, and lesions and lymphoid tissue sent for 'confirmation' by culture – a less sensitive technique of diagnosis, but one that allows

molecular typing of the causative *M. bovis* and so aids epidemiological investigation of outbreaks. Other molecular-based methods are being currently developed in order to detect active infection rather than simply the immune response, such as nested PCR (Araújo et al., 2014), immune-magnetic separation-based methods (Stewart et al., 2013) and loop-mediated isothermal amplification (LAMP)(Zhang et al., 2011).

Vaccination of cattle is not allowed under EU trade regulations, pending the development of a test that can distinguish between vaccinated and infected cattle or a vaccine that allows identification of infected animals (DEFRA, 2015a).

In the UK, control of bTB includes cattle testing and removing positive animals from the herd. Depending on location and risk of infection, tests are performed from every six months to every 4 years. Positive holdings are placed under movement restrictions, increased biosecurity measures, while in high risk and edge areas, control of wildlife reservoir (badger) populations through culling and vaccination are being trialled (DEFRA, 2014, APHA(WALES), 2015b).

The economic impact of bovine tuberculosis is remarkable: just in Great Britain, the number of animals slaughtered as reactors or contacts has increased from 5,685 in 1998 to 30,220 in 2013, of which 16,840 (55%) were found in South West England, Devon being the county with the highest herd incidence (Baker, 2015). The efforts to address bovine TB cost the UK tax-payer over £80 million a year in compensation, cattle testing, research and surveillance.

In the past, control of TB was achieved largely by testing and culling cattle. However, since the 1980s, the incidence of bTB in cattle has increased, and an epidemic that appeared first in the southwest of England has now reached the northwest. Badgers are believed to be involved in the current epidemic, making control more difficult. Transmission from badgers to cattle has been demonstrated experimentally (Little et al., 1982), but the precise details of transmission in the field are poorly understood. Most transmission appears to be indirect – either in pasture or by badgers contaminating cattle feed (Hutchings and Harris, 1999, Garnett et al., 2002, Bohm et al., 2008); however, this assumption is still controversial as some studies have found that badgers avoid farmyards and cattle do not graze areas contaminated by badger urine or faeces (Benham and Broom, 1991, Mullen et al., 2015). According to some researchers, the presence of infected badger setts near cattle-occupied areas can increase the risk of bTB in the cattle (Martin et al., 1997). Interventions, such as badger culling, can reduce TB in local cattle (Krebs et al., 1997, Griffin et al.,

2005, Krebs, 2011), but also increase the prevalence of infection in the remaining badgers and in badger populations surrounding the culled area through perturbation of normal stable, and territorial, badger populations. This perturbation effect may then cause spill over of the infection into cattle in areas surrounding the cull area (Donnelly et al., 2003, Donnelly et al., 2007, Woodroffe et al., 2009).

If cross-species transmission in endemic areas is complicated, then the drivers of expansion of the cattle epidemic are even less well understood. The leading edge of the epidemic could be in cattle, with spill over into badgers, in badgers, with spill over into cattle, or a combination of the two. That genotypes (spoligotypes) of *M. bovis* tend to have regional distributions, might suggest not one national epidemic but several local ones – although whether the regional foci of these spoligotype-specific mini-epidemics is cattle, badgers or something else is not known. This relatively poor understanding of the drivers of transmission and the expanding epidemic make the design of control policies difficult. That one of the control policies is the culling of badgers, makes control highly controversial politically, with entrenched views based not just on scientific evidence but the place badger hold in popular culture in the UK (Morris, 1987, Lodge and Matus, 2014).

Sections 2.1 and 2.2 in chapter 2 of this thesis describe stakeholder-led studies of TB in badgers in Cheshire and Greater Manchester, on the northern edge of the bTB epidemic in cattle.

1.2.2. Cryptosporidium spp.

Cryptosporidium is an apicomplexan parasite with a single host life cycle comprising six major developmental phases (Figure 1.1):

1. The release of infective sporozoites from the Oocyst (excystation),
2. Asexual multiplication within the host cell (merogony),
3. Formation of microgametes and macrogametes (gametogony),
4. Union of macrogametes and microgametes (fertilization),
5. Formation of environment resistant oocysts (oocyst wall development) and
6. Formation of infective sporozoites (sporogony) .

The taxonomy of cryptosporidia used to be based on oocyst morphometry, host and site of infection; however, the development of DNA sequencing techniques has led to a new and more extensive classification of *Cryptosporidium* species (Thompson and Monis, 2004, Xiao, 2010).

Currently there are 26 accepted species of *Cryptosporidium*, 17 of which have been found infecting humans (Table 1.5) (Ryan et al., 2014). However, not all strains/genotypes found in non-human animals are zoonotic: the development of high resolution DNA characterisation methods has shown that some genotypes of previously thought zoonotic species are in fact, specific to non-human animals (Xiao, 2010, Li et al., 2014).

Transmission occurs through the faecal-oral route, and it is considered that *Cryptosporidium spp.* is responsible for almost 20% of human water-associated diarrhoeal disease outbreaks (Karanis et al., 2007). In 2012, over 9,000 cases of cryptosporidiosis were reported within the European Union and the countries of the European Economic Area, with 68% of these in the UK alone (Fournet et al., 2013, ECDC, 2014).

In livestock, particularly cattle, sheep and goats, *Cryptosporidium* infections cause diarrhoea in young animals. In cattle, infection is mainly due to *C. parvum, C. bovis, C. andersoni*, and *C. ryanae*. In sheep, the species identified include: *C. parvum, C. andersoni, C. suis, C. xiaoi, C. fayeri, C. ubiquitum, C. scrofarum*. In goats, although less studied, species identified include: *C. parvum, C. ubiquitum, C. xiaoi.* In New Zealand *C. hominis* has also been reported in all three species of livestock (Abeywardena et al., 2014), and in sheep and a goat in the UK (Giles et al., 2009, Connelly et al., 2013).

In pigs, the most common species found are *C. suis* in pre-weaned piglets and *C. scrofarum* in older pigs (Nemejc et al., 2013). However, *C. muris* and *C. parvum* have also been reported. As with other livestock species, the clinical signs seem to be related to age, with younger animals more likely to have diarrhoea (Morgan et al., 1999).

In horses, *C. parvum, C. erinaceae* and *C. hominis* have been detected, although clinical disease and prevalence is still unclear (Majewska et al., 2004, Laatamna et al., 2013, Laatamna et al., 2015).

C. canis and *C. felis* are the main causes of cryptosporidiosis in dogs and cats respectively, although *C. parvum* and *C muris* have also been reported. As with livestock, clinical disease seems to be limited to young or immunocompromised animals (Abe et al., 2002, Ballweber et al., 2009, Lucio-Forster et al., 2010).

As might be expected, *Cryptosporidium* spp can also infect wildlife. For example, rabbits have been found to harbour *C. cuniculus*, and disease (diarrhoea) has been seen in young animals (Shi et al., 2010, Zhang et al., 2012). In cervids, *C. parvum, C. ubiquitum, C. bovis, C. ryanae* and *C. hominis* have been described (Garcia-Presedo et al., 2013, Nolan et al., 2013).

Cryptosporidium species recognised in birds include *C. meleagridis, C. baileyi* and *C. galli*, as well as a number of distinct avian genotypes and *C. parvum* and *C. hominis*. In turkeys, parrots, chickens, cockatiels and red-legged partridges *C. meleagridis* has been associated with enteritis and mortality, and is also considered an emerging human pathogen (Xiao and Fayer, 2008). *C. baileyi* is associated mainly with respiratory disease with high morbidity and mortality and to lesser extent with renal disease, particularly in broiler chickens, although it has been reported in a wide range of birds. *C. galli*, is most likely cause of gastrointestinal disease in finches, chickens, parrots, flamingos, cardinals and rhinoceros hornbills (Ng et al., 2006, Ryan, 2010).

Cryptosporidium in fresh water and marine water fish has been associated with stomach or intestinal infection. Three species (*Cryptosporidum molnari*, *Cryptosporidium nasorum* and *Cryptosporidium* scophthalmi) have been suggested based on oocyst morphology (Hoover et al., 1981, Alvarez-Pellitero and Sitja-Bobadilla, 2002, Alvarez-Pellitero et al., 2004,), but currently, only *C. molnari* is officially recognised as a species (Ryan et al., 2014).

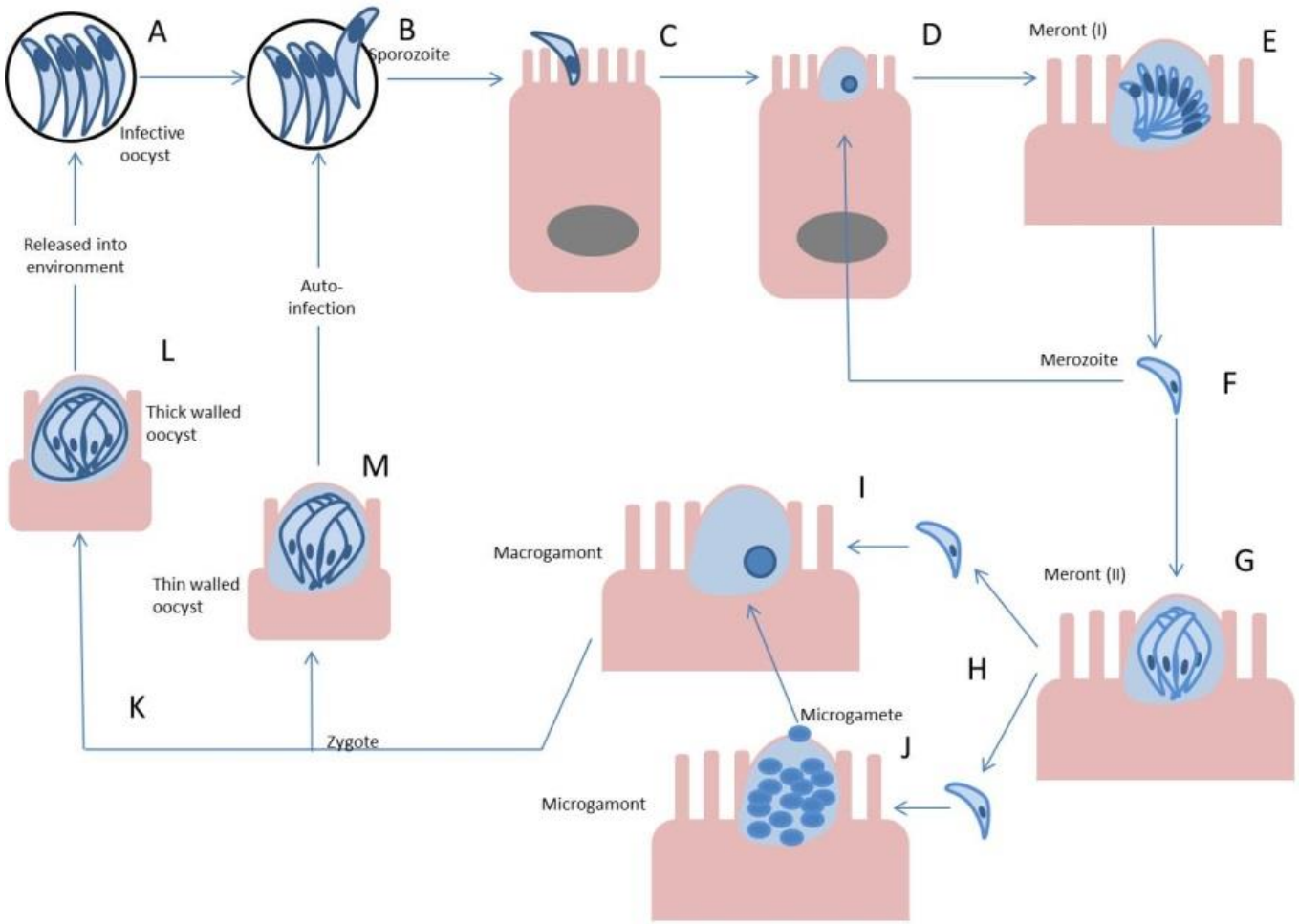

Figure 1.1 *Cryptosporidium* life cycle Infective oocysts are ingested (A) and sporozoites are released (encystation) (B). Sporozoites invade intestinal cells (C) and transform into trophozoites which undergo merogony to transform into a type I meront with eight merozoites (D). The merozoites are released and they reattach to adjacent epithelial cells where they transform into type I or type II meronts with four merozoites (F, G). These merozoites are released and attach to epithelial cells and initiate gametogony (H) to produce either macrogamonts or microgamonts (I, J). Each microgamont produces up to 16 microgametes by nuclear division, which are then released to fertilize a macrogamont and produce a zygote (K). Each zygote undergoes two asexual cycles of sporogony and produce an oocyst with thin wall (M) which will then auto – infect the host or an oocyst with thick wall which will be released into the intestinal lumen and excreted through faeces to infect a new host (L).(Bouzid et al., 2013).

Table 1.6. Species currently accepted of *Cryptosporidium* and their main hosts. * species reported in humans and non-human animals (Ryan et al., 2014). ** species reported only in humans.

Species name	Main host
Cryptosporidium muris*	Rodents
Cryptosporidium wrairi	Guinea pigs
Cryptosporidium felis*	Cat
Cryptosporidium serpentis	Snakes and lizards
Cryptosporidium meleagridis*(IIIa, IIIb, IIIc, IIId, IIIe, IIIf, IIIg)	Birds and humans
Cryptosporidium parvum * (IIa*, IIb**, IIc**, IId*, IIe**, IIf, IIg, IIh, IIi, IIk, III, IIm, IIn, IIo)	Ruminants
Cryptosporidium baileyi	Birds
Cryptosporidium varanii	Lizards
Cryptosporidium andersoni*	Cattle
Cryptosporidium canis*	Dogs
Cryptosporidium molnari	Fish
Cryptosporidium hominis*(Ia, Ib, Id, Ie, If*, Ig, Ih, Ii, Ij)	Humans
Cryptosporidium galli	Birds
Cryptosporidium suis*	Pigs
Cryptosporidium bovis*	Cattle
Cryptosporidium fayeri*(IVa,IVb, IVc, IVd, IVe, IVf)	Marsupials
Cryptosporidium fragile	Toads
Cryptosporidium macropodum	Marsupials
Cryptosporidium ryanae	Cattle
Cryptosporidium xiaoi*	Sheep and goats
Cryptosporidium ubiquitum(XIIa*, XIb*, XIIc*, XIId*, XIIe, XIIf)	Ruminants, rodents, primates
Cryptosporidium cuniculus	Rabbits
Cryptosporidium tyzzeri*	Rodents
Cryptosporidium viatorum*	Humans
Cryptosporidium scrofarum*	Pigs
Cryptosporidium erinacei*	Hedgehogs and horses

In marine mammals, *C. hominis* and *C. parvum* have been identified in various pinnipeds and cetacean species, which would suggest contamination of marine environments from anthropogenic activities (Appelbee et al., 2005).

Amphibians and reptiles have been reported with cryptosporidiosis, causing gastrointestinal disease and death in the case of some snakes; however, few studies have been conducted in these species. *C. serpentis* and *C. varanii* are capable of infecting lizards and snakes, while *C. fragile* has been found in toads (Jirku et al., 2008, Ryan et al., 2014, Zahedi et al., 2016).

Control measures to avoid outbreaks of cryptosporidiosis in humans are based on water treatment with UV radiation, which is currently the most widely used method for oocyst inactivation, and where possible, removal of wildlife and livestock from areas where water destined for human consumption is collected. Despite these interventions, however, outbreaks are still common, even in industrialised countries.

Chapter 3.1 describes a study to investigate the possible sources, in particular wildlife sources, of *Cryptosporidium* oocysts found in a drinking water reservoir in North Wales.

1.2.3. ***Giardia spp*.**

Giardia is a flagellated protozoan, with a single host life cycle and environmentally resistant infective cysts. *Giardia* possesses a simpler life cycle than *Cryptosporidium* comprising four major stages (Figure 1.2):

1. Ingestion and excystation of trophozoites triggered by low pH in stomach,
2. Multiplication by binary fusion of trophozoites in the proximal small intestine of the host,
3. Encystation of trophozoites in the jejunum and
4. Expulsion of cysts through faeces, enabling infection of the next host.

There are currently six recognised species of Giardia, of which only *Giardia duodenalis* (previously known as *Giardia lamblia* or *Giardia intestinalis*) has been found to infect humans (Feng and Xiao, 2011). Further genetic characterisation reveals *G. duodenalis* to be divisible into seven sequence-based assemblages (A-G), of which only A and B are considered human pathogens (Table 1.7) (Thompson et al., 2000, Feng and Xiao, 2011). Furthermore, assemblage A has been found to have three subtypes (AI, AII and AIII): AI and AII are associated with human and other animal infections (AII has been found mostly in humans) while AIII is found almost exclusively in wild hoofed animals (Caccio and Ryan, 2008, Xiao and Fayer, 2008, Monis et al., 2009). Assemblage B can also be divided into subtypes, of which BIII and BIV have been found mainly in humans and to a lesser extent in wild and domestic animals (Sprong et al., 2009).

Transmission, as for *Cryptosporidium*, is mainly by ingestion of infective cysts in food and water, although person-to-person transmission is also possible (Hoque et al., 2002). In 2012, over 16,000 cases of giardiasis were reported within the European Union and the countries of the European Economic Area, with 26% of these in the UK (Fournet et al., 2013, ECDC, 2014).

In humans, infection can cause diarrhoea, either acute or chronic, but in many cases is asymptomatic. Malabsorption and decreased growth can be consequences of infection, particularly in children. Other symptoms include vomiting, abdominal cramps, bloating and nausea. Diagnosis is usually by direct observation of cysts or trophozoites in faeces and treatment is mainly with nitroimidazole derivatives (Thompson et al., 1993, Thompson, 2000,CFSPH, 2012).

In other mammals, clinical signs are mainly seen in young animals, in which mucoid diarrhoea with weight loss seem to be the most common signs, particularly in ruminants (Geurden and Olson, 2011), while in pigs infection appears to be almost always asymptomatic (Geurden et al., 2010).

Perhaps the most famous wildlife species involved in *Giardia* zoonotic transmission is the American beaver (*Castor canadensis*): in fact, giardiasis in North America was commonly referred to as 'beaver fever'. Beavers are frequently implicated in human water-borne outbreaks in N. America (Dykes, 1980, Daly et al., 2010).

Marine mammals have also have been shown to harbour *Giardia sp.* but the clinical impact as well as the zoonotic potential of infection in these hosts is yet to be established (Measures and Olson, 1999).

In birds, malodorous diarrhoea, dehydration, pruritus and feather pulling with alopecia have been associated with *Giardia* infection (CFSPH, 2012). In most reptiles, infection seems to be non-pathogenic, however, a *Giardia* sp. has been linked to gastroenteritis in some snakes (Scullion and Scullion, 2009). *G. duodenalis* (assemblages A, B and E) and *G. microti* have been found in fish, and some species of marine mammals, however, the effect of these infections in these hosts has not been studied (Lasek-Nesselquist et al., 2008, Yang et al., 2010).

Control in water of *Giardia*, as well as *Cryptosporidium* , is through disinfection treatments in water works and plants; successful removal or inactivation of cysts can be affected by the concentration of cysts in raw water (Teunis et al., 1997).

Chapter 3.2 describes the use of animal and water samples positive for *Giardia spp*. collected in chapter 3.1 in order to investigate their zoonotic potential through molecular typification and phylogenetic characterisation.

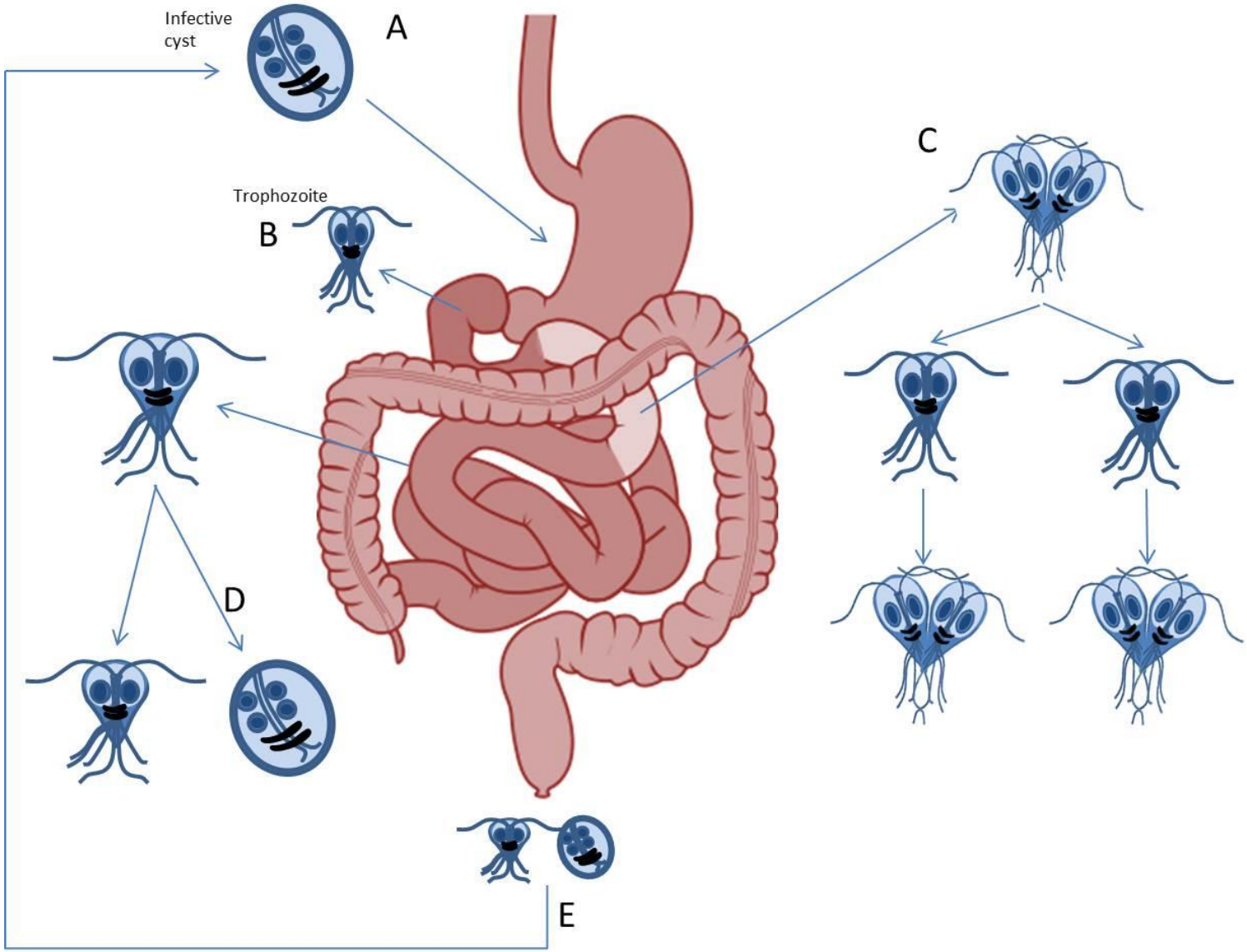

Figure 1.2. The life cycle of *Giardia spp*. Cysts found in the environment are ingested; the low pH of the stomach promotes the release of the trophozoite (encystation) (A, B). Trophozoites attach to the small intestine where multiplication by binary fusion takes place producing malabsorption and diarrhoea in some hosts (C). The contact with biliary salts results in encystation of the trophozoites (D) which are then excreted from the host through faeces (E).(Adam, 2001).

Table 1.7. Species of *Giardia* and genetic assemblages. Genetic genotyping has removed certain species and re-classified them into *G. duodenalis* assemblages. (*) previous nomenclature. (**) assemblages identified in human cases (Monis et al., 2009)

Species (previous name)	Assemblage	Host
Giardia duodenalis	A**	Humans, primates, dogs, cats, livestock, rodents, other wild mammals
Giardia enterica*	B**	Humans, primates, dogs, other wild mammals
Giardia canis*	C	Canids
Giardia canis*	D	Canids
Giardia bovis*	E	Cattle and hoofed livestock
Giardia cati*	F	Cats
Giardia simondi*	G	Rats
Giardia agilis		Amphibians
Giardia muris		Rodents
Giardia psittaci		Birds
Giardia ardeae		Birds
Giardia microti		Rodents

1.2.4. ***Campylobacter spp.***

The Family Campylobacteriaceae comprises the genera *Arcobacter*, *Campylobacter* and *Sulfurospirillum*, of which *Campylobacter spp,* particularly *C. jejuni* and *C. coli* are the most important in terms of public health, having been implicated in human enteritis cases since the late 1970's (Skirrow, 1977).

Campylobacters are non-spore forming, spiral, Gram-negative bacteria. Although they appear only to multiply within animal hosts and are often thought of as not surviving well in the environment (Jones, 2001), *C. jejuni* has been shown to survive and maintain metabolic activity at 4°C for at least 14 days (Hazeleger et al., 1998), and can be readily isolated from soil and water environments. In laboratory conditions, most campylobacters grow at a narrow temperature range (30-46°C), and they require microaerobic (5% O2, 10% CO2 and 85% N) conditions. These conditions are believed to be an adaptation to the low oxygen concentrations found in the gastrointestinal tract of wild and domestic animals, particularly poultry which have been considered their original host (Park, 2002, Garenaux et al., 2008). There are 26 species currently accepted, of which 17 have been associated with human disease (Table 1.8).

Campylobacter is a major cause worldwide of diarrhoeal disease with more than 90 million cases reported every year and approximately 21,000 related deaths (WHO, 2015). It accounts for around 8.4% of the total burden of diarrhoea worldwide and is considered the fourth most common infectious cause of enteritis after rotaviruses, typhoid fever and cryptosporidiosis (WHO, 2012a), with *C. jejuni* and *C. coli* responsible for 80% and 10% of campylobacteriosis cases, respectively (OIE, 2015b). In the UK there are an estimated 500,000 clinical cases of campylobacteriosis each year (Tam et al., 2012) while in the US, food-borne campylobacteriosis affects around 2.5 million people each year (Mead et al., 1999).

C. jejuni has a low infective dose (500-800 cfu) in otherwise healthy human adults, causing illness within 48 hours of ingestion (Robinson, 1981, Black et al., 1988). However this may be affected by age of the patient, concurrent medical conditions such as diabetes mellitus or the use of proton-inhibitor drugs (anti-acids) which increase susceptibility to campylobacter infection (Neal et al., 1996, Neal and Slack, 1997, Tam et al., 2009, Nichols et al., 2012). Socio-economic factors such as education and income can also influence both susceptibility and contact with contaminated food. According to a study conducted in Denmark, cases of campylobacteriosis are not linked to poverty or

limited resources but rather to an increase in income, which allows people access to foreign travel as well as fresh products rather than cheaper frozen ones (freeze-thawing is quite effective at killing campylobacters) (Simonsen et al., 2008).

Symptoms of campylobacteriosis in people vary, but they may include diarrhoea with or without blood, abdominal pain, fever, nausea, malaise, and more rarely vomiting (Allos, 2001). In severe cases there can be intestinal haemorrhage (Patel et al., 2013), toxic megacolon (Anderson et al., 1986) and haemolytic uremic syndrome (Chamovitz et al., 1983). Mortality associated with campylobacter infections is low (0.1-8.8%) although influenced by age - mortality is higher in children under 5 and adults over 65 years old - and pre-existing risk factors such as HIV/AIDS, liver cirrhosis, cancer or diabetes (Smith and Blaser, 1985, Pacanowski et al., 2008, Barton Behravesh et al., 2011). Clinical signs can last up to ten days and are usually self-limiting, although there are three major sequelae linked to campylobacter infections: reactive arthritis (ReA), Guillain-Barré syndrome and non-paralytic variant Miller-Fisher syndrome and irritable bowel syndrome (IBS), as well as other functional gastrointestinal disorders (FGD) (WHO, 2012a).

In many other animal species enteric infection is common without any obvious disease and with very similar carriage rates between healthy and diarrhoeic animals (Prescott and Bruin-Mosch, 1981, Svedhem and Kaijser, 1981, Manser and Dalziel, 2009). In dogs and cats, *Campylobacter* infection, may cause diarrhoea but clinical signs are often associated with concurrent GI infections (eg with coronovairus). In cattle and sheep, some species of *Campylobacter* (*C. fetus, C. sputorum* and sometimes *C. jejuni*) have been also associated with abortion and infertility. In pigs *C. mucosalis* and *C. hyointestinalis* are commonly implicated in gastroenteritis, but *C. coli* and *C. jejuni* usually cause no obvious clinical signs.

Similarly diarrhoea, lethargy, hepatitis and weight loss are some of the signs related to *C. jejuni* infection in birds, including poultry and pet species, but again most infections are clinically unapparent. Other animal species susceptible to *Campylobacter* infections include ferrets, mink, non-human primates and rodents, where disease is dependent on each species but can present as mucoid diarrhoea, emesis, anorexia and fever.

Wild birds have been shown to carry potentially zoonotic species of *Campylobacter,* and some birds could potentially distribute infection over large areas. However, their real contribution to human disease has been put into question by the use of modern sequencing techniques that have shown

that some of the genotypes carried by wild birds are, in fact, not the same as those found in human cases or livestock (Hughes et al., 2009, Hepworth et al. 2011).

Campylobacter-associated enteritis is still an expensive disease: it has an estimated cost of £500 M per year in the UK and USD$ 3.4 M in the US (Buzby and Roberts, 1997, Humphrey et al., 2007). The European Food and Safety Authority (EFSA) has developed an integrated approach consisting of risk assessment and risk management measures to protect consumers' health. The UK has developed a similar campaign called 'acting on Campylobacter together' to reduce the levels of bacteria that reach the consumer (https://www.food.gov.uk/news-updates/campaigns/campylobacter/actnow).

The main source of human campylobacteriosis – perhaps responsible for 60-80% of cases, in Europe and North America is poultry, although red meat products and the environment are also frequent sources of infection. The control of infection in poultry is based on biosecurity in the farm environment and, in some countries, disinfection of carcasses after slaughter: however the contamination rate among carcasses at retail in the UK remains high with just over 70% of tested chickens found positive for *Campylobacter*, 19% of which were found to harbour over 1,000 CFU/g, additionally, in almost 7% of samples, *Campylobacter* was also isolated from the outer packaging (Jorgensen et al., 2015). Effective vaccines for poultry are not available although they are the subject of much research (Neal-McKinney et al., 2014, Kobierecka et al., 2016).

Chapter 4 investigates the potential role of wild birds in the ecology of campylobacteriosis on farms. Newly hatched chicks do not have campylobacter infections, so the infection must be introduced later in their lives. Previous surveys have shown infection to be common in some wild bird species and many environmental isolates are believed to come from birds, therefore it is believed that wild birds might either be reservoirs of infection for poultry or receivers of poultry strains from waste etc. Wild birds on two farms; a poultry and a dairy farm, were sampled as part of a larger programme investigating campylobacters in the environment.

Table 1.8. Currently accepted *Campylobacter* species, their sources and association with human disease.

Campylobacter species	Isolation source	Human disease associated	References
C. avium	Poultry	None reported	(Rossi et al., 2009)
C. canadensis	Cranes	None reported	(Inglis et al., 2007)
C. coli	Livestock, wild birds, humans, dogs	Gastroenteritis	(Platts-Mills and Kosek, 2014)
		septicaemia	(Kist et al., 1984)
		meningitis	(Thomas et al., 1980,Blaser et al., 1986, Man, 2011)
C. concisus	Humans, pets	Gastroenteritis	(Nielsen et al., 2013, Vandenberg et al., 2013)
		periodontal desease	(Tanner et al., 1987, Moore and Moore, 1994)
		abscesses	(de Vries et al., 2008)
		Crohn's disease	(Mahendran et al., 2011)
		Ulcerative colitis	(Mukhopadhya et al., 2011)
C. corcagiensis	Lion-tailed macaques	None reported	(Koziel et al., 2014)
C. cuniculorum	Rabbits	None reported	(Zanoni et al., 2009)
C. curvus	Humans, dogs,	Gastroenteritis	(Abbott et al., 2005)
		periodontal disease	(Moore and Moore, 1994)
		Abscesses	(Han et al., 2005)
C. fetus (subsp. Fetus, venerealis, testudium)	Cattle, sheep, reptiles, humans, dogs	Gastroenteritis	(Wagenaar et al., 2014)
		septicaemia	(Tu et al., 2004)
C. gracilis	Humans, dogs	Periodontal disease	(Macuch and Tanner, 2000, Siqueira and Rocas, 2003)
		abscesses	(de Vries et al., 2008)
C. helveticus	Cats, dogs	None reported	(Moser et al., 2001, Engvall et al., 2003)
C. hominis	Humans	None reported	(Lawson et al., 2001)
C. hyointestinalis subsp. hyointestinalis	Pigs, cattle, humans	Gastroenteritis	(Edmonds et al., 1987, Lawson et al., 1999, Kim do et al., 2015)
C. hyointestinalis subsp. lawsonii	Pigs, humans	Gastroenteritis	(Gorkiewicz et al., 2002)
C. iguaniorum	Reptiles	None reported	(Gilbert et al., 2015)
C. insulaenigrae	Marine mammals, humans	Gastroenteritis	(Chua et al., 2007)
		septicaemia	
C. jejuni (subsp. jejuni, doylei)	Humans, livestock, wild birds, poultry, dogs	Gastroenteritis	(Lawson et al., 1999, Platts-Mills et al., 2014)
		Guillain-Barre syndrome,	(Kuroki et al., 1993, Kuwabara and Yuki, 2013)
		meningitis	(Thomas et al., 1980, Goossens et al., 1986, Man, 2011)
		septicaemia	(Tee and Mijch, 1998, Wolfs et al., 2001)
C. lanienae	Cattle, pigs, humans	Enteritis	(Lévesque et al., 2016)
C. lari (subsp. lari, concheus)	Wild birds, poultry, dogs, cats, shellfish, humans	Gastroenteritis,	(Tauxe et al., 1985, Broczyk et al., 1987, Chiu et al., 1995)
		septicemia	(Bruneau et al., 1998, Werno et al., 2002)
C. mucosalis	Pigs, dogs	None reported	(Lawson et al., 1975, Chaban et al., 2010)
C. peloridis	Molluscs,humans	None reported	(Debruyne et al., 2009)

Table 1.5 (continuation)			
C. rectus	Humans, dogs	Periodontal disease	(Rams et al., 1993, Moore and Moore, 1994, Macuch and Tanner, 2000)
		abscesses	(Siqueira and Rocas, 2003, Han et al., 2005, de Vries et al., 2008)
C. showae	Humans, dogs	Periodontal disease	(Macuch and Tanner, 2000, Figueredo et al., 2008)
		abscesses	(de Vries et al., 2008)
C. sputorum (bv sputorum, paraureolyticus)	Cattle, pigs, humans	Gastroenteritis	(Vandenberg et al., 2006, Lindblom et al., 2009)
		Abscesses	(On et al., 1992)
		septicaemia	(Tee et al., 1998)
C. sputorum bv. faecalis	Sheep, cattle	None reported	(On, 1996)
C. subantarticus	Albatrosses, penguins	None reported	(Debruyne et al., 2010)
C. troglodytes	Chimpanzees	None reported	(Kaur et al., 2011)
C. upsaliensis	Humans	Gastroenteritis	(Lawson et al., 1999, Bullman et al., 2012, Couturier et al., 2012)
		Septicemia	(Nakamura et al., 2015)
C. ureolyticus	Humans, horses	Gastroenteritis	(Bullman et al., 2011, O'Donovan et al., 2014)
		abscesses	(Duerden et al., 1982)
		Ulcerative colitis	(Mukhopadhya et al., 2011)
C. volucris	Black headed gulls, humans	Bacteremia	(Kweon et al., 2015)

1.3. The study and its aims

This thesis describes and discusses four (potentially) zoonotic pathogens, found in livestock but that also have, or might have, a wildlife component to their epidemiology. None are classical emerging infections – although the geographic expansion of the bovine TB epidemic in the UK might fit the definition of an EID. In each case, the precise role of wildlife in the epidemiology of the disease is unclear and controversial. The same organisms have been found in people, livestock and wildlife, but questions remain about cross-species transmission. It is important to understand the role of wildlife in such disease systems in order to focus interventions where they might have benefit to public and animal health, and to maintaining biodiversity, and, importantly, optimise the use of increasingly scarce resources. Each chapter focusses on a different disease system, but the general discussion at the end of this thesis will attempt not only to bring together some of the common findings and differences between each disease system but discuss some of the cultural and political issues encountered in undertaking such studies.

The overarching aim of the work described in this thesis was, therefore:

> **To investigate, using four infectious diseases of economic and public health importance in the UK as study systems, the role of wildlife in the epidemiology of multihost, zoonotic infections.**

More specific aims included:

> To determine the feasibility of using road-killed sampling, and a stakeholder network, to investigate TB in badgers at the edge of the English bovine TB epidemic in cattle. And, if the sampling was successful, the possibility of interrogating those data to investigate:
>
> a. The presence/absence and approximate or estimate prevalence of bTB in badgers; and
> b. Any spatial and/or genotypic relationships between infected badgers and infected cattle.
>
> To determine the role, if any, of wildlife in an observed annual peak in *Cryptosporidium* oocysts in a potable water reservoir, and, if oocysts were detected in wildlife and environmental samples:
>
> c. Determine the relationship of cryptosporidia from different sources to each other and to known genotypes; and
> d. Determine the likely zoonotic potential of cryptosporidia from these different sources.
>
> To use the samples collected for the *Cryptosporidium* study to investigate also *Giardia duodenalis* in wildlife, livestock and the environment, and, if cysts were detected:

e. Determine the relationship of *Giardia* from different sources to each other and to known genotypes; and
f. Determine the likely zoonotic potential of *Giardia* from these different sources.

To determine the rates of infection of wild birds on a poultry and a dairy farm with *Campylobacter spp*, and if found to

g. Compare these isolates with those found in a recent study of livestock and the environment on both farms, and with other studies of wild birds; and
h. Thereby investigate the potential role of wild birds in the epidemiology of on-farm campylobacteriosis.

2. Bovine tuberculosis (bTB) and the European badger (*Meles meles*)

2.1. Tuberculosis in Cheshire badgers – a survey of road-traffic killed badgers on the edge of the UK epidemic

2.1.1. Introduction

Bovine tuberculosis (bTB) in cattle in Great Britain is concentrated in Southwest England and South Wales, but has been gradually spreading northwards in England (DEFRA, 2014). Cattle and people can be infected with several members of the *Mycobacterium tuberculosis* (MTB) complex, but most recent bovine cases in England and Wales have been caused by *M. bovis*. Bovine tuberculosis is of importance both as a zoonosis (although human infection in England and Wales is rare, largely owing to the pasteurisation of most milk) and for its effects on international trade and therefore the economic and social cost of its control. The epidemiology and control of bTB in the UK is complicated by infection in badgers, which can maintain bTB and potentially transmit it back to cattle (Corner et al., 2014).

In 2013 the Department of Environmental, Food and Rural Affairs (DEFRA) produced a 'Strategy for achieving an Officially Bovine Tuberculosis Free status (OTF status) for England by 2025' (DEFRA, 2014). The strategy is based on the categorisation of England into three areas: the High Risk Area (HRA), concentrated in the South West, West Midlands and East Sussex, where bTB is endemic with repeated breakdowns and a high incidence of bTB in wildlife (badger) populations; the Low Risk Area (LRA), which comprises the North and East of England, where prevalence is very low, breakdowns are relatively short majorly linked to animals introduced from higher risk areas and there is no recognised wildlife reservoir; and the Edge Area which is the boundary between the HRA and the LRA and has variable incidence with a tendency of outward spreading from the HRA towards the LRA. Most of Cheshire belongs to this Edge area (Fig. 2.1.1).

Until recently, there were only sporadic outbreaks of bTB in cattle in Cheshire, one of the most important centres of the British dairy industry. On the basis of both epidemiological investigations of these outbreaks and the genetic analysis (spoligotyping) of *M. bovis* isolated from them, most such outbreaks were thought to be largely the result of the importation of cattle from endemic areas (Gilbert et al., 2005). However, since around 2010 more frequent outbreaks have been reported in

herds, particular in south eastern Cheshire, which by 2013 was on the northernmost edge of the national epidemic. The role of badgers in the epidemiology of bTB in Cheshire is unknown: previous studies in Cheshire, all undertaken more than a decade before this study, found no or only few infected animals, with those few infected badgers being found in south eastern Cheshire (Krebs et al., 1997, Atkins and Robinson, 2013).

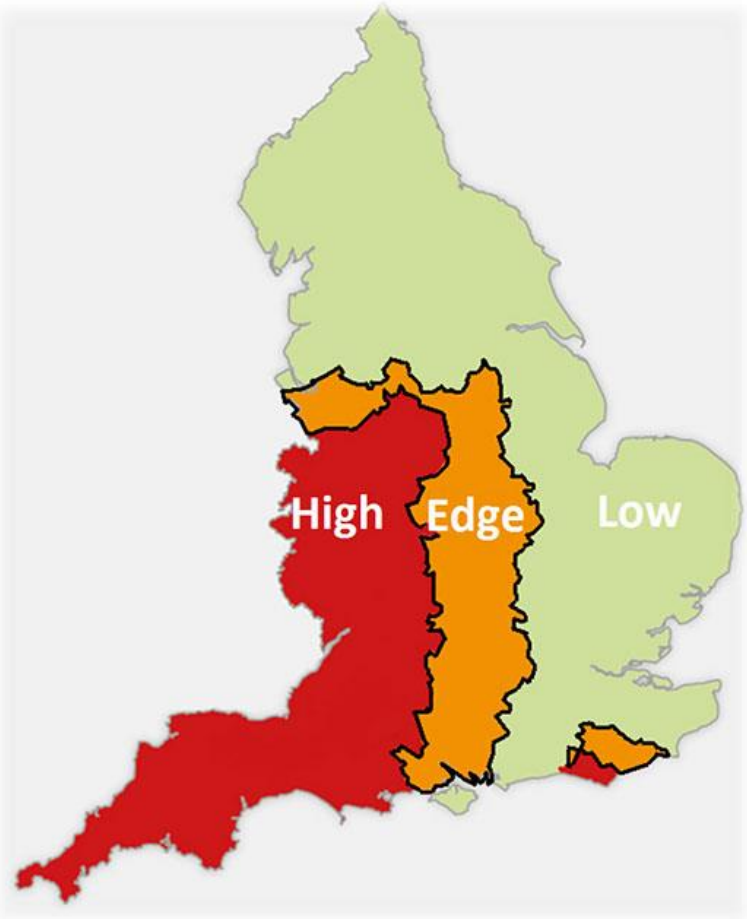

Fig 2.1.1 Counties and the TB risk categorisation according to the DEFRA Strategy (DEFRA, 2014)

An important aim of the national bTB control strategy is to prevent further expansion of the epidemic (DEFRA, 2014), and it is recognized that this will require much greater understanding of the transmission of *M. bovis* at the edge of the epidemic. While there have been many studies of bTB epidemiology in what are now endemic areas, particularly the south west of England, the role of badgers (and other wildlife) in the expansion of the epidemic is largely unknown: expansion of the epidemic in cattle might be due to cattle-to-cattle transmission (including over long distances through cattle trading) or due to transmission between badgers, with subsequent cross species transmission in either or both directions, or the expansion of the epidemic might result from a more complex combination of both processes.

This chapter describes a stakeholder-led 'feasibility study' of bTB in road traffic-killed badgers in and around Cheshire, undertaken during 2014 and 2015: the aim of the study was both to determine if bTB could be detected in badgers and, perhaps more importantly, if such an approach might be feasible as a means of wildlife surveillance for TB across a wider area. At the time of planning the project, bTB in cattle in much of Cheshire was regarded as sporadic and it was intended that the study investigate bTB in badgers ahead of the epidemic front. In the event, 2014 saw a doubling of bTB outbreaks in Cheshire herds, over a wider area than in previous years, and data from cattle surveillance in Cheshire in 2014 have therefore been included in this study for comparison with the findings in badgers.

2.1.2. Methods

Badgers and sample collection

The survey was undertaken through the Cheshire TB Eradication Group, an informal body funded in 2013 comprising farmers, veterinarians (both in private practice and working for the Animal and Plant Health Agency, APHA), Cheshire Wildlife Trust and local badger protection groups, the National Farmers Union (NFU) and other stakeholders including Local Authorities and livestock markets. The group met regularly in order to share information and experiences, and to discuss bTB and its control in Cheshire.

At every meeting, I gave members of the group collection kits and asked them to collect (following the recommendations of the risk assessment included in every kit) fresh badger carcasses seen on roads while travelling as part of their normal daily activities in Cheshire or, if farms or holdings crossed county borders, just over the borders into neighbouring counties (Figure 2.1.2). An additional 5 carcasses were donated by the Royal Society for the Prevention of Cruelty of Animals (RSPCA) or local veterinary surgeons after they had to be euthanized or died after admission to their medical centres.

Carcasses collected from areas beyond those set out above are described in a separate section of this chapter (2.2). A formal risk assessment, guidance and training on safe collection, handling and transport were given to the members of the group, as well as "collection kits" comprising submission forms, heavy duty plastic bags, secure ties, gloves and masks (Figure 2.1.3).

Carcasses estimated at being no more than two days dead, were delivered to the Liverpool Veterinary School, where, after storage at 4°C for no more than 24 hours, a standardised post mortem examination and tissue sampling were undertaken inside a Containment Level 3 (CL3) laboratory. Carcasses with autolysis suggesting death more than three days prior to necropsy, or with open abdomens through trauma and so likely to be heavily contaminated were not used in the study. Furthermore, with only a few exceptions (eg. carcasses from wildlife rescue organisations), only carcasses clearly involved in road traffic accidents were accepted into the study. As badgers are protected by UK law, contributors were warned that any evidence of illegal trapping or killing would be reported to the police: no such evidence was seen.

Sex, age and type of road where the carcass was found were recorded. The age (estimated by dentition, body mass and size) was recorded as juvenile (< 1 year) or adult (> 1 year)(Harris et al., 1992). Roads where carcasses were found were classified as A (major roads that provide large-scale transport links between areas), B (roads intended to connect areas and A roads to smaller roads) or C (smaller roads that connect A and B roads and often connect housing states or villages to the rest of the network), according to the Cheshire (east and west) council (https://www.whatdotheyknow.com/request/list_of_roads_67) and using Google maps. Both residential and unnamed roads (both intended for local traffic) were included in class C for the purpose of this analysis. No carcasses were collected from motorways (M roads) due to safety and legal reasons.

Figure 2.1.2. Cheshire borders and limits for carcass admittance in the study.

Figure 2.1.3 Collection kits

Samples collected during the post-mortem examination included: any visible lesions compatible with TB in any organ, superficial, thoracic and abdominal lymph nodes, lung lobes, spleen, liver and kidneys. For culture, each lesion was processed separately but tissue pools were created from non-lesion material: a 'lung pool' of lung lobe samples, a 'thoracic pool' of bronchial and mediastinal lymph nodes, an 'abdominal pool' comprising liver, spleen, kidneys and hepatic and mesenteric lymph nodes, a 'head and neck' pool of parotid, mandibular, retropharyngeal and cervical lymph nodes, and a 'carcass pool' of prescapular, axillary, and superficial inguinal lymph nodes. Smears of lesion material were stained for acid-fast bacteria using a modified Ziehl-Neelsen method (Engelkirk and Duben-Engelkirk, 2008), and tissues with visible lesions were examined histologically. Individual tissue samples were stored at -80°C if needed for further study.

Attempted isolation *of M. bovis* from badgers

All tissue processing and culture was undertaken in the Leahurst containment level 3 (CL3) facility (University of Liverpool). Tissue pools were gently ground with sterile sand and 2-3ml of phosphate buffer solution, then mixed with an equal amount of 5% oxalic acid for 5 to 10 minutes to reduce contamination with bacteria other than mycobacteria (Yajko et al., 1993, Corper and Uyei, 1930). Pooled samples were inoculated on to both Stonebrink Selective agar (BD Diagnostics, Oxford) and Lowenstein-Jensen with pyruvate slopes (Media for Mycobacteria Ltd. Penarth) and incubated at 37°C for a minimum 12 weeks (Cousins, 2012). Cultures were examined weekly for the appearance of colonies characteristic of *M. bovis*.

Characterisation of *M. bovis* isolates from badgers

DNA was extracted from colonies by heating a suspension of 1-2 colonies in 100 µl DNA-free water at 80°C for 30 minutes and/or by use of Qiagen DNA extraction kits using the manufacturer's instructions. Spoligotyping was carried out at APHA, Weybridge. Spoligotyping was also undertaken by Dr. Ben Swift at the University of Nottingham using DNA microarray technology (Alere Technologies, Germany). Briefly, extracted DNA from colonies was diluted 1:1000 into sterile distilled water (SDW). PCR was performed using biotinylated primers which were used to amplify the DR region of the *M. bovis* genome (Kamerbeek et al., 1997). The amplified DNA was then hybridized onto ArrayStrips using the *M. bovis* spoligotyping array kit (Alere) according to Ruettger et al. (2012).

Cattle data

The locations of Officially TB Free Withdrawn Status (OTFW) premises, as well as Officially TB Free Suspended (OTFS) premises within Cheshire tested between 2014 and 2015 was provided by APHA (Pers. Comm.). OTFW premises are those where skin test-positive cattle were culled and TB was 'confirmed' post mortem by visible lesions or culture. OTFS are holdings where 'reactors' were not 'confirmed' as having TB at post mortem examination. Both OTFW and OTFS are breakdowns – ie both have bTB, but OTFW may be at a more advanced stage of disease progression.

Analytic and statistical approaches

Prevalence determination was not the intended aim of this study as the precise population of badgers in Cheshire is both unknown and varies greatly across the area (Cheshire Wildlife Trust, unpublished data). However, an estimated prevalence was calculated from the sampled badgers, as in previous studies (Abernethy et al., 2003, Goodchild et al., 2012), based on the assumption that the carcasses collected were sufficiently representative of the overall population.

Age, sex, season and type of road (A, B or C) where carcasses were found were also analysed to identify associations with bTB infection in badger carcasses. These analyses were performed using chi square tests when the expected frequencies were 5 or more for each cell. When more than 20% of the data had expected frequencies of 5 or less, Fisher's exact probability test was performed, using the Freeman-Halton's extension when the variable consisted of more than 2 categories.

Kulldorf's spatial scan statistic (SaTScan™ ver. 9.4.2) was used to investigate the presence of clusters of bTB- positive badgers in Cheshire using the Bernoulli model. The default parameters were used as suggested in the user's guide. (Kulldorff and Nagarwalla, 1995, Kulldorff, 1997, Kulldorff et al., 2005, Kulldorff, 2015).

In addition, the distance between both bTB positive and negative badger carcasses and positive cattle holdings (OTFW and OTFS) was measured, and the Mann-Whitney-Wilcoxon test for non-normally distributed data was used to evaluate the significance of the medians using R version 3.3.1 (R Core Team, 2014).

The number of bTB positive holdings within a 5km radius of each collected badger was recorded. The significance of the odds and likelihood of positive badgers being found near positive farms was assessed using Fisher's exact test, as performed in a previous study (Goodchild et al., 2012). Analysis was done using the Vassar stats website for statistical computation (http://vassarstats.net/).

The location of badger carcasses and cattle holdings was mapped using the "Rgoolgemaps" package (Loecher, M., 2014) in R version 3.3.1 (R Core Team, 2014), with locations jittered for presentation as it was agreed with the stakeholders that precise locations would not be disclosed for reasons of confidentiality.

2.1.3. Results

Overall, 94 badger carcasses were collected from within or very close to the borders of Cheshire (Figure 2.1.4). The female/male sex ratio was 0.88 (53% males and 45% females). During post-mortem examination, two pseudohermaphrodite badgers were identified, one juvenile and one adult. For further analyses these two badgers were considered as males as they were most likely male pseudohermaphrodites according to Bigliardi *et al*. (2011).

The age ratio was 2:1 adult: juvenile (66% and 34% respectively) (Figure 2.1.5). Badger carcasses were collected mostly in spring (35% between March and May) and autumn (32% between September and November), compared to winter (16% in February 2014 and December to February 2015) and summer (17% between June and August). A total of 45 carcasses were found on A roads, 7 on B roads and 42 on C roads.

Overall, MTB-complex bacteria were isolated from 20 badgers, all of which were confirmed by genotyping as *M. bovis* spoligotype 25. Hence, the estimated prevalence of bTB among badgers in this study was 21.3% (CI 95% 14.2 to 30.6). The prevalence among juvenile badgers was 15.6% (5/27) and amongst adults 24.2% (15/47). The prevalence among males was 15.4 % (8/44) and among females was 28.6% (12/30). Of the positive carcasses, eight were found on A roads (8/45), 10 on C roads (10/42) and 2 on B roads (2/7). There was no statistically significant association between sex and age and the risk of bTB infection of badgers (Table 2.1.1). The prevalence observed throughout the year was relatively constant with no evidence of statistically significant seasonal variations in infection risk (Table 2.1.1). No statistically significant difference in the probability of infection was found between the different roads types (Table 2.1.1).

One badger had characteristic TB lesions disseminated in lymph nodes and other organs, including lung, liver, spleen and kidney. The badger was an adult and based PM body mass and dental wear it was considered to be over 3 years old. Seven other badgers, four culture-positive and three culture-negative, had similar age-related dental wear; however, due to limited methods for age determination they were all classified as adults (> 1 year old). Smears from visible lesions stained positive for acid fast bacteria, and histological examination of fixed lung lesions demonstrated characteristic granulomatous foci with numerous acid fast bacilli (Figure 2.1.6). All typical tuberculous lesions yielded *M. bovis* in culture. Only one carcass had macroscopic lesions, therefore, latency was estimated at 95% (19/20).

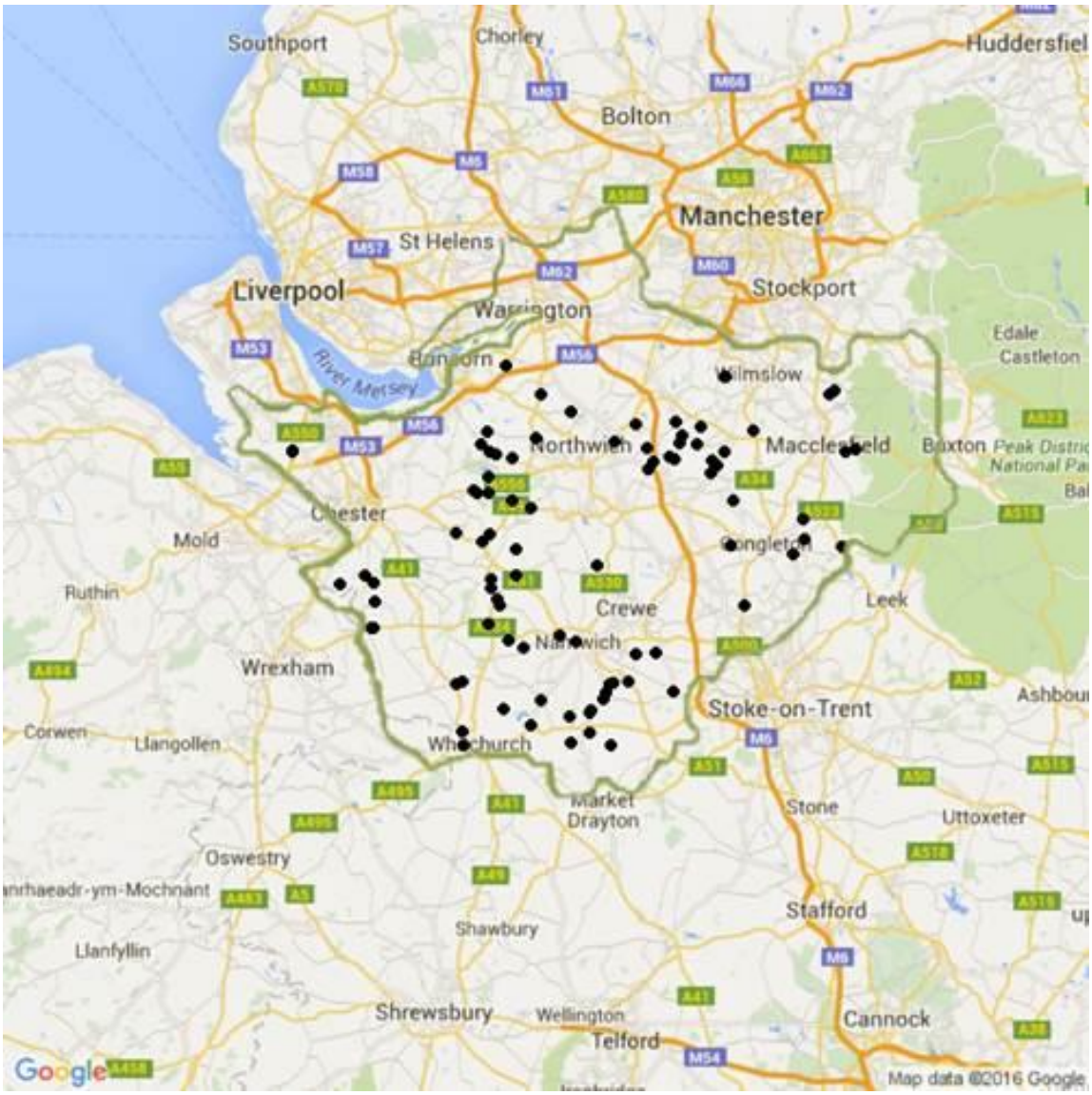

Figure 2.1.4. Location of badgers included in the study.

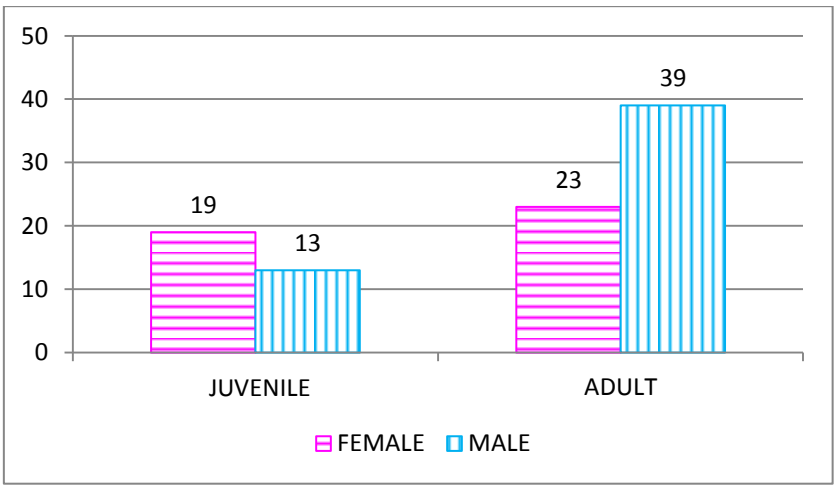

Figure 2.1.5. Proportion of badger carcasses collected by sex and age

Table 2.1.1. Prevalence, confidence intervals and statistical tests results according to age, sex, season and road categories (X^2 or Fisher's exact probability test. *Freeman-Halton's extension).

	Positive	Negative	Prevalence	95% Confidence limits Lower	Upper	X^2 / FEPT	P
Juvenile	5	27	15.6%	6.9	31.8	0.92	0.3
Adult	15	47	24.2%	15.2	36.2		
Males	8	44	15.4%	8.3	28.5	2.4	0.1
Females	12	30	28.6%	16.4	41.8		
Winter	4	12	25.0%	10.2	49.5		0.98*
Spring	7	26	21.2%	10.7	37.8		
Summer	3	12	20.0%	7.05	45.2		
Autumn	6	24	20.0%	9.5	37.3		
A roads	8	37	17.8%	9.3	31.3		0.68*
B roads	2	5	28.6%	8.2	64.11		
C roads	10	32	23.8%	13.5	38.5		

Overall, 36 pools from 20 badgers were positive; the tissue pools most frequently culture-positive were the 'carcass' lymph nodes (ie pools of axillary, prescapular, and inguinal lymph nodes), and head and neck lymph nodes, with 33% each of all positive pools. Thoracic lymph nodes and abdominal pools (mesenteric and hepatic lymph nodes, liver, kidney and spleen) accounted for 17% and 14%, respectively. Only the carcass with visible tuberculous lesions had a positive lung pool (3%) (Table 2.1.2). Of the positive carcasses, 50% had only one positive tissue pool, 40% had between 2 and 3 positive tissue pools and only 10% had 4 or more positive pools.

Overall, the badger carcasses came from 31 different towns and parishes belonging to Cheshire (East and West) and areas near the borders. Of these locations, 11 had bTB positive badgers. The area with the most submissions was Knutsford, which accounted for almost 13% of carcasses, of which 20% (2/10) had bTB, while Congleton was the source of 8% of submitted carcasses but 86% (6/7) were positive. The total submissions and results can be seen in Table 2.1.3 and Figure 2.1.7.

Figure 2.1.8 shows the location of both OTFW and OTFS cattle holdings along with the (jittered) locations of culture positive and culture negative badger carcasses. These collection sites, although not uniformly distributed, included most regions of the county. Figure 2.1.8 might also suggest that badger carcasses were particularly submitted from areas in which bovine TB outbreaks had, or were, occurring, an impression reinforced by informal conversation with multiple stake-holders.

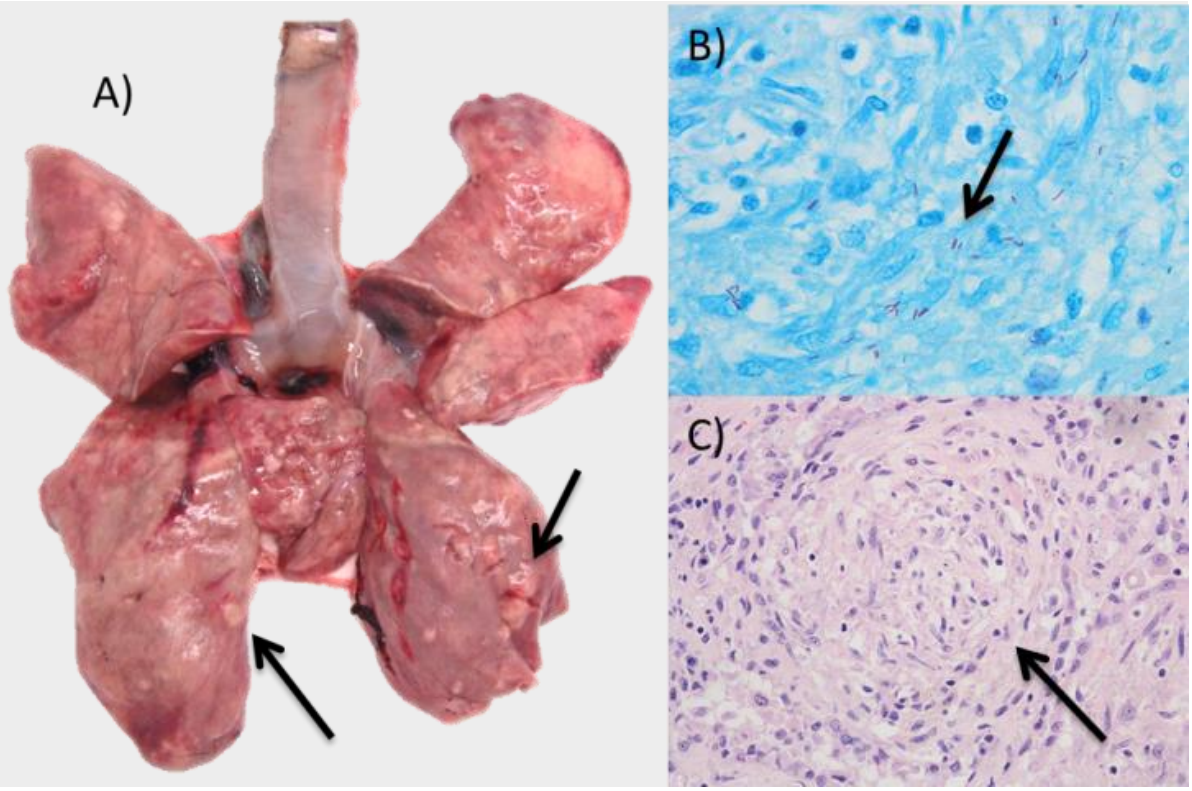

Figure 2.1.6. Characteristic TB macroscopic lesions in lung tissue(A). Mycobacteria inside the granulomatous areas can be seen in red (B) (ZN 100x) and the granulomatous foci surrounding the affected area can be observed in fixed lung tissue (C) (HE 10x)

Table 2.1.2. Number and type of positive cultures by tissue pool (head, carcass, abdomen, thorax and lungs)

Positive pools	Number of carcasses	Percentage	Positive pools				
			Head	Carcass	Abdomen	Thorax	Lungs
One	10	50%	2	3	2	3	0
Less than 4	8	40%	8	7	1	1	0
4 or more	2	10%	2	2	2	2	1

Kulldorf's spatial scan statistic suggested a 7.79 km radius cluster of positive badgers in the eastern part of the study area, near the Derbyshire border (Figure 2.1.9). The relative risk of disease within this cluster was 6.29 (p=0.032) and was considered a true cluster using the Gini coefficient.

The median of the distance between a positive badger and a holding with a TB outbreak (either OTFW or OTFS) was 1.1km compared with 1.8km between a negative badger and a positive holding. This difference was not statistically significant (p=0.06) (Figure 2.1.10). Badgers were significantly more likely to be bTB positive if there were more than six cattle outbreaks registered within a 5km radius, (Fisher's exact test p=0.01), and the odds of a badger being bTB positive was 4.2 times

greater if 6 or more cattle breakdowns were found within a 5km radius of the carcass (95% confidence interval 1.3 to 13.9).

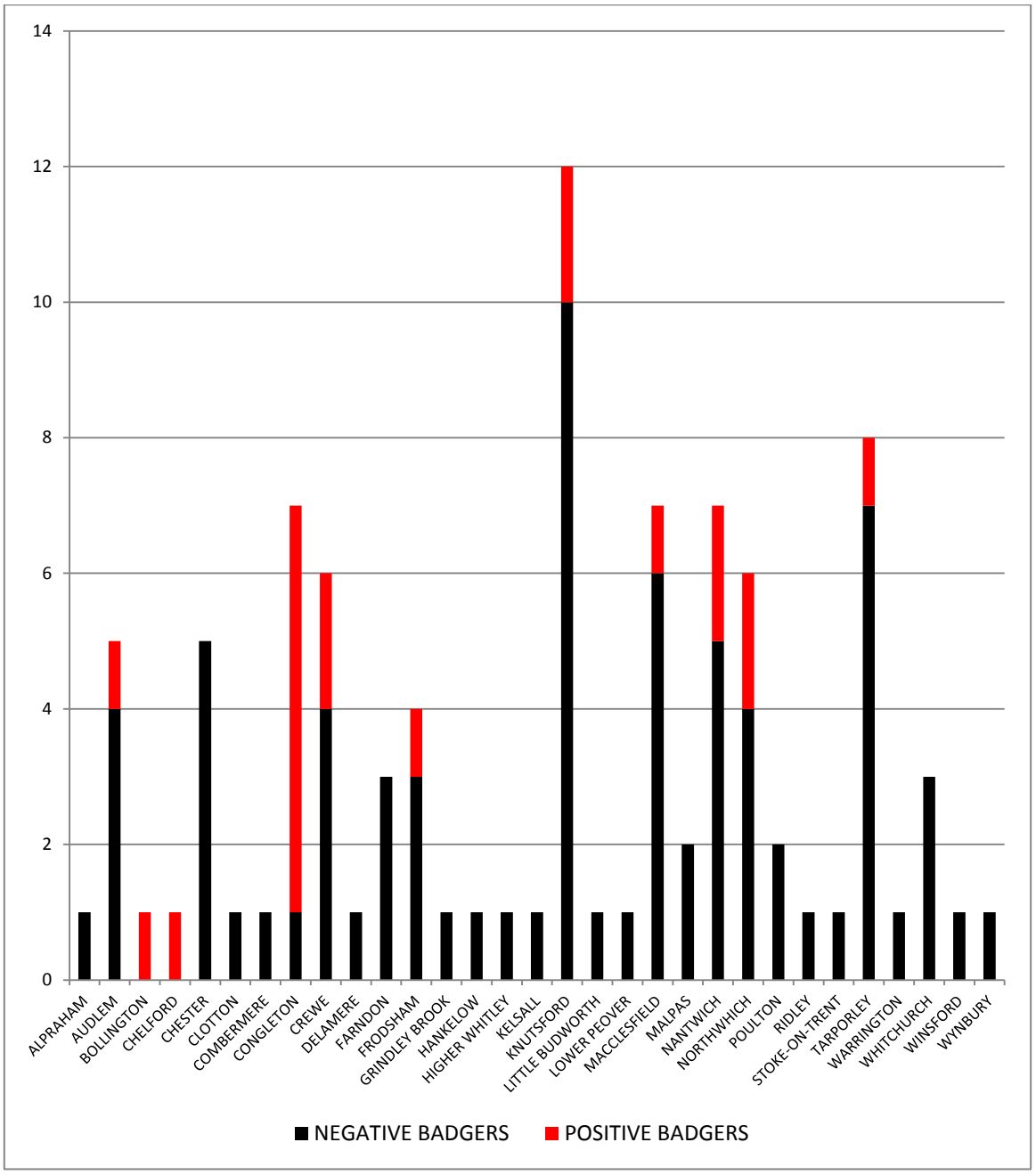

Figure2.1.7. Areas where carcasses were found and their bTB status. Negative (black) and positive (red) carcasses are shown by town or parish.

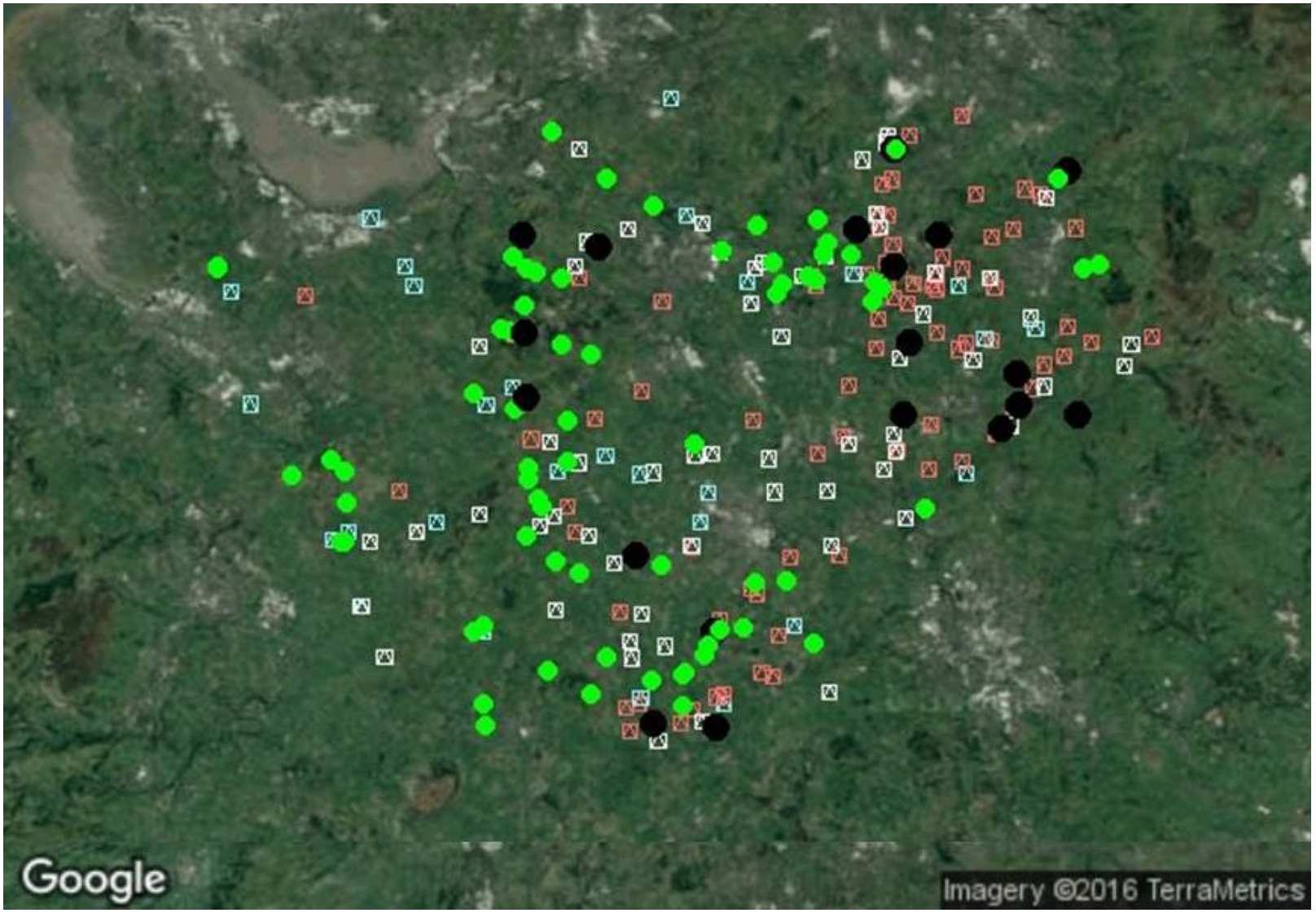

Figure 2.1.8 Location of positive (black) and negative (green) badger carcasses compared to cattle holdings testing positive for bTB: OTFS (white) and OTFW with confirmed SP25 (orange) and other confirmed spoligoytpes (blue).

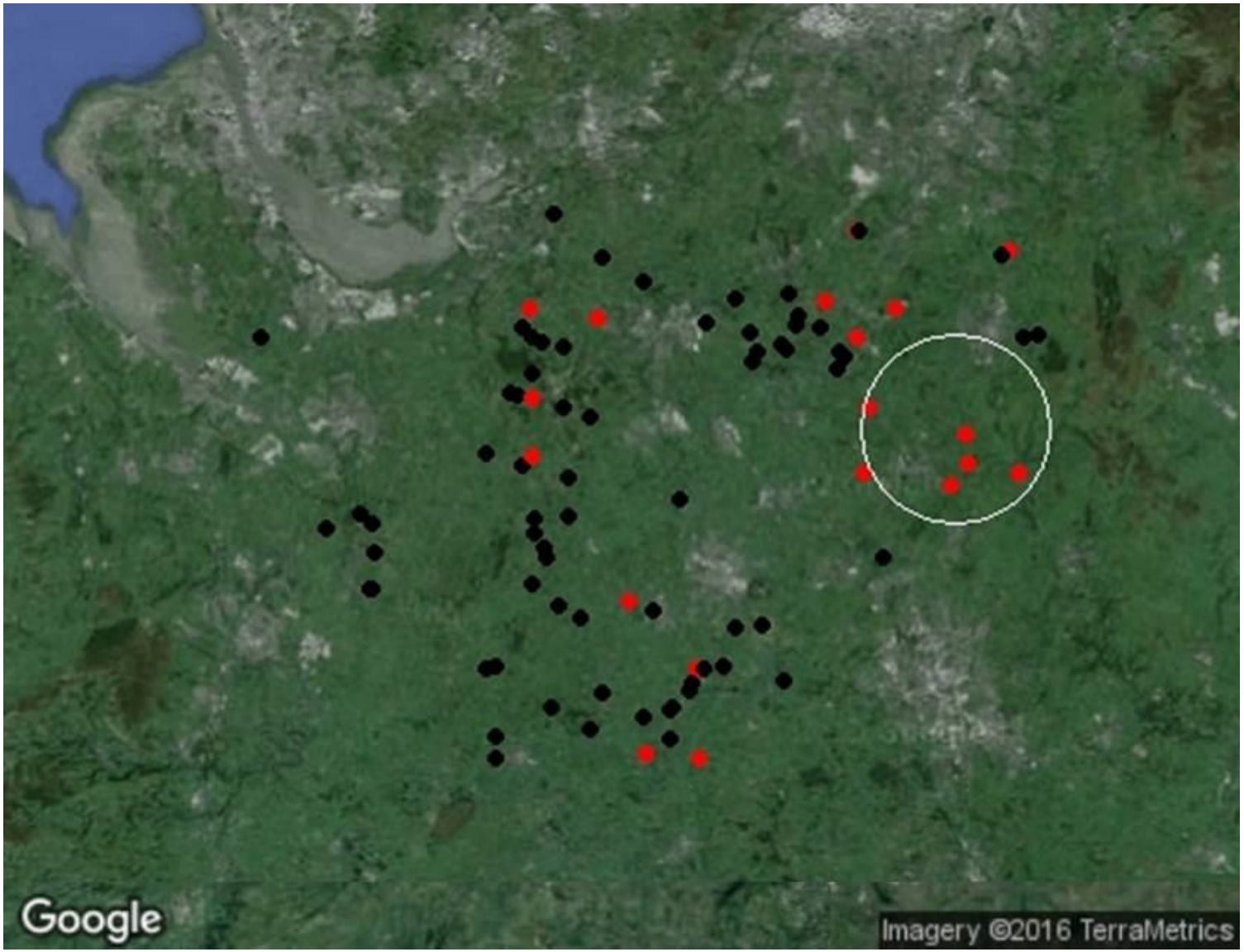

Figure 2.1.9. Kuldorff´s spatial scan statistic (SatScan™) shows a 7.79 km radius cluster of badgers near the Derbyshire border in the Congleton area.

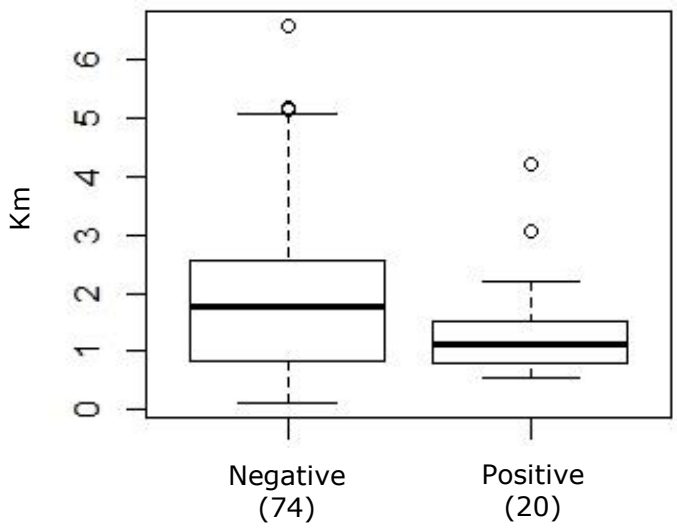

Figure 2.1.10 Median distance between negative (1.8 km) and positive (1.1 km) badgers to OTFW or OTFS holdings.

Table 2.1.3. Areas from which badger carcasses were obtained, bTB status and spoligotype of isolates

LOCATION	NEGATIVE BADGERS	POSITIVE BADGERS	TOTAL BADGERS	% OF SUBMISSIONS	SPOLIGOTYPE
ALPRAHAM	1	0	1	1.06%	SP25
AUDLEM	4	1	5	5.32%	SP25
BOLLINGTON	0	1	1	1.06%	SP25
CHELFORD	0	1	1	1.06%	SP25
CHESTER	5	0	5	5.32%	SP25
CLOTTON	1	0	1	1.06%	SP25
COMBERMERE	1	0	1	1.06%	SP25
CONGLETON	1	6	7	7.45%	SP25
CREWE	4	2	6	6.38%	SP25
DELAMERE	1	0	1	1.06%	SP25
FARNDON	3	0	3	3.19%	SP25
FRODSHAM	3	1	4	4.26%	SP25
GRINDLEY BROOK	1	0	1	1.06%	SP25
HANKELOW	1	0	1	1.06%	SP25
HIGHER WHITLEY	1	0	1	1.06%	SP25
KELSALL	1	0	1	1.06%	SP25
KNUTSFORD	10	2	12	12.77%	SP25
LITTLE BUDWORTH	1	0	1	1.06%	SP25
LOWER PEOVER	1	0	1	1.06%	SP25
MACCLESFIELD	6	1	7	7.45%	SP25
MALPAS	2	0	2	2.13%	SP25
NANTWICH	5	2	7	7.45%	SP25
NORTHWHICH	4	2	6	6.38%	SP25
POULTON	2	0	2	2.13%	SP25
RIDLEY	1	0	1	1.06%	SP25
Stoke-on-Trent	1	0	1	1.06%	SP25
TARPORLEY	7	1	8	8.51%	SP25
WARRINGTON	1	0	1	1.06%	SP25
WHITCHURCH	3	0	3	3.19%	SP25
WINSFORD	1	0	1	1.06%	SP25
WYNBURY	1	0	1	1.06%	SP25
TOTAL BADGERS	74	20	94	100.00%	

2.1.4. Discussion

Tuberculosis caused by *M. bovis* is the main cause of re-emerging TB in domestic livestock, non-human primates and free-living and captured wildlife globally (El-Sayed et al., 2016). Between 2005 and 2010, cattle herds infected with *M. bovis* were detected in 109 countries (Ramos et al., 2014), which represents 56% of countries worldwide.

The main aim of this study was to evaluate the use a road kill survey to determine the presence of *M. bovis* in badgers in Cheshire. Isolation of *M. bovis* from badger carcasses was possible and the overall estimated prevalence of *M. bovis* isolated from road-killed badgers in the Cheshire area was 21.3% or 20/94 (CI 95% 14.2 to 30.6). This prevalence estimate is, perhaps, higher than expected from an area on the edge of the national bTB epidemic in cattle, as it is comparable with the prevalence estimates in dead badgers of 18% and 20% in Worcestershire and Gloucestershire respectively (ISGCTB, 2007). This might be the result of some of the biases involved in a road-kill study, or it may indicate that Cheshire should not be regarded as an 'Edge' county. Certainly this prevalence is higher than that found in the RTA surveys in Cheshire (and other areas) between 1972 and 1990, which found only one positive badger out of 389 collected (0.26%) (Atkins and Robinson, 2013).

Road traffic and 'found dead' surveys have previously been used to study several wildlife diseases (Case, 1978, Anthony et al., 1990, Aarissorensen, 1995, Flegr et al., 2002, Nelder and Reeves, 2005, Richini-Pereira et al., 2008, Santos et al., 2011, ,). They can be influenced by a range of spatial and temporal factors that include: animal density and behaviours, road type and use, seasonality, collection convenience and safety, and engagement by those reporting or collecting carcasses (Santos et al., 2011, Collinson et al., 2014). It is likely that similar biases applied to this study. For example, there was a possible concentration of carcasses submitted from areas with bTB outbreaks in cattle, and this might reflect the majority of carcasses having been submitted by farmers who might in turn have a particular interest in having local badgers tested (the category of stakeholder of each submitted carcass can be found in Appendix C). Larger scale studies would be needed to examine such potential biases, and also any relationship between badger carcasses submitted with types of road (in this study it was not possible to sample badgers from major roads on which stopping is illegal), local badger population size and ranges, type of landscape, and other factors effecting local stakeholder engagement.

There are currently no published data on the distribution or density of badgers in Cheshire, however, unpublished data collected by Cheshire Wildlife Trust during its TB badger vaccination campaign in 2013 and 2014 found an average estimated density of 14 to 19 badgers per km^2 in 2013 and 16 to 22 badgers per km^2 in 2014, with areas ranging from 0.72 badgers per km^2 (1 badger in 1.37 km^2) to 10.5 badgers per km^2. These estimates may be inflated by their being based on surveys of areas with known badger populations, but suggest densities not dissimilar to those found in the South West areas of England in 2010, where densities ranged from 1.5 – 4.8 per km^2 (Parrott et al., 2011).

As found in previous studies not based on road-killed badgers (Macdonald and Newman, 2006, Aarissorensen, 1995), the ratio of males and females sampled in this study approached 1:1. Adults (>1 years) represented 66% of the sampled population while young animals (<1 year) represented only 34%. These results also agree with previous studies based on non-road killed samples (Cheeseman et al., 1981, Delahay et al., 2000). This suggests that the demographics of found-dead/road killed carcasses broadly reflect those of the source populations.

A previous analysis of road traffic mortality in badgers found spring and autumn peaks in death rate, (Davies et al., 1987), with the spring peak comprising females and males and the autumn peak largely of males. Similar results were found in this study. However, the prevalence of bTB remained constant, at around 20%, in each season. This suggests that any future time-constrained survey for bTB in badgers might be best focussed on the spring and autumn months.

The relationship between the locations of OTFW and OTFS holdings and probability of badgers nearby being bTB positive in this study was significant ($p=0.01$) only when six or more bTB breakdowns happened within a 5 km radius. The difference between the direct median distance between positive holdings and positive or negative carcasses was not significant ($p=0.06$). However, the observed p value, which approached the critical threshold of 0.05, suggests this hypothesis warrants further investigation in the future.

Abernethy et al. (2003) found no significant difference in risk of TB for herds adjacent to infected carcasses in Northern Ireland, unlike Goodchild et al. (2012) who found in a recent study, a strong association between infection status in dead badgers and presence of a single cattle holding breakdown (CHB) within a 5km radius. The lack of association between positive badgers and less than six holdings might indicate either that the badger and cattle outbreaks are unrelated or that

infection in badgers depends on the burden of infection in cattle, i.e. it requires a relatively high number of cattle breakdowns to increase the probability of badgers becoming infected.

Kuldorff's spatial statistical analysis seeks to detect the presence of clusters of infection by constructing a series of circular or elliptic windows of varying size over a specified area and comparing the relative risk (RR) of infection within these windows with RR outside of them (Pfeiffer *et al*. 2008). The radii of these windows vary to include a range of the population from 1% to the maximum percentage of the population at risk (maximum spatial cluster size) specified in the parameters, which in this case was 50%. The maximized likelihood of infection of each window is evaluated and compared; the one with the maximum likelihood is considered the most likely cluster as it is the one less likely to have occurred by chance (Kulldorff, 2015). The Bernoulli model was used because, unlike the Poisson model, it estimates the relative risk based on a comparison of cases and controls as Boolean variables, while in the Poisson model cases are compared to the background population data. The cluster detected in the area of Congleton, suggests that the risk of badgers in this area being bTB positive is unlikely to be simply due to random or chance events. In contrast, fluctuations in the density of bTB positive badgers in other areas are consistent with random variation. It is important to notice that potential bias in the location of the cluster could be due to location and density of roads within and outside the window, as well as probability of a dead badger being picked up from that area. Further discussion on these biases can be found in the limitations of the study section.

Kulldorff's spatial statistical analysis was used as an exploratory tool to find possible clusters of infected badgers in Cheshire; and as such, it was determined that the default parameters should be used. Some of these parameters have limitations, particularly the maximum spatial cluster size, as it is debated that bigger cluster sizes can hide small homogenous clusters within big heterogenous ones and smaller cluster sizes can miss the regional-level clusters and therefore influence the sensitivity of the SatScan™ results (Chen et al., 2008). Another limitation of the SatScanTM software is that it cannot evaluate clusters with arbitrary shapes (Yao et al. 2011). Using the latest version of the software can overcome some other limitations, like lack of visual geographical output (version 9.4 can produce KML files for Google Earth and shapefiles for GIS software) and the use of a hierarchical approach to determine the most likely cluster and the secondary clusters (version 9.3 onwards uses the Gini coefficient to determine which clusters are best to report) (Han et al., 2016).

In 2014, the most commonly isolated genotype (spoligotype) found in cattle breakdowns in Cheshire was SP25, however, SP17 and SP35 had also been isolated from the West and South West (near the Welsh border) areas of the county. Some of the SP17 breakdowns (5) were traced to cattle bought from Cumbria (APHA, 2015b). All the isolates from badgers were of spoligotype SP25. The lack of genetic diversity in badger isolates, despite the variation in cattle genotypes might point towards an independent epidemic within the badger population. Whole genome sequencing of geographically-related isolates from cattle and badgers might enable better interrogation of the issue of whether or not transmission occurred between cattle and badgers in Cheshire and possibly even the direction of transmission.

In this study, the majority of positive cultures were from 'head and neck' and 'carcass' pools of lymph nodes (33% for both). Some previous studies found that most isolates were found from lung and thoracic lymph nodes suggesting that, in badgers, tuberculosis is primarily a respiratory disease resulting from aerosol infection (Murphy et al., 2010, Corner et al., 2011). However, not all studies collected as great a diversity of lymph nodes as in this study. Corner et al (2012) found a high frequency of infection of axillary lymph nodes but a low incidence in more peripheral lymph nodes, and suggested that isolation from external and peripheral lymph nodes could be due to extra-pulmonary dissemination of infection or a secondary pathogenic pathway through the mucosa of the upper respiratory tract. Although some authors have reported bite wounds and external lesions as common and possible source of *M. bovis* bacteria (Cheeseman and Mallinson, 1981, Clifton-Hadley et al., 1993, Macdonald et al., 2004) we found no badger carcass presented signs of bite wounds in this study. However, it is possible, in some cases, that the severity of the collision or lesions suffered during the traffic accident could have masked the presence of bite wounds.

Parrish et al. (1998) defined 'latency' as pathogenic mycobacteria contained within the host without the development of macroscopic lesions, the lack of these type of lesions in most of the carcasses examined agrees with previous studies which suggest that latency is the most common form of tuberculosis in badgers (Corner et al., 2012), although the prevalence of latency obtained in this study (95%) was higher than previously reported which ranged from 30 to 80% (Gallagher and Clifton-Hadley, 2000, Murphy et al., 2010, Corner et al., 2012). The only carcass with disseminated macroscopic lesions belonged to the adult category but with marked signs of being older than 3 years which could suggest that older badgers are more likely to present lesions, perhaps due to immunosuppression associated to old age, or to the impact of chronic infection. Unfortunately, the

small number of carcasses (8) that could fit into this subcategory, did not allow for conclusions to be drawn regarding the link between age and lesions.

There is some evidence that bTB infection might affect behaviour in badgers (Cheeseman and Mallinson, 1981), which could in turn influence the risk of infected animals being hit by vehicles. If such a behavioural effect exists, it may be expected that the prevalence of infection estimated through road-kill surveys would be biased, although whether towards an over-estimate ('ill' badgers move about more and/or can't avoid cars) or an under-estimate ('ill' badgers don't venture out but hide within setts). However, most infected animals in this study had no visible tuberculous lesions and appeared at post mortem examination to have been healthy immediately prior to being killed. Previous studies have suggested that 'latent' bTB has minimal effect on the life histories of badgers (Cheeseman et al., 1988, Wilkinson et al., 2000) with some authors even suggesting a 'late recovery' phase in the badger TB pathogenesis (Gallagher et al., 1998). Other animals with MTB complex infections (e.g. voles with TB caused by *M. microti*) also show few signs of altered behaviour or changes in life history even with advanced lesion development (Cavanagh et al., 2002, Cavanagh et al., 2004). These observations, combined with the overall similarity of the prevalence and demographic results in this study with those in previous studies not reliant on road kill data, suggest that while care must be taken when drawing conclusions, useful information on bTB in badgers can be gained from engaging a range of stakeholders in future road kill studies.

Previous studies found that prevalence of infection in badgers was higher in adults than cubs (Woodroffe et al., 2009), and in males than females (Gallagher and Nelson, 1979, Cheeseman et al., 1981, Cheeseman et al., 1989, ISGCTB, 2007, Goodchild et al., 2012), although, in most cases, the difference was not statistically significant and the larger number of males infected with TB was attributed to wounds due to territory fights, larger home ranges and more daring behaviours. This study also found no statistically significant difference in infection rates among males and females or adults and juveniles. However, unlike previous studies, a larger number of females than males were infected. Delahay et al. (2000) suggested that when a female was infected it increased the chances of cubs in the same sett to be infected too.

Limitations to the study

Relying on a network of people to obtain the carcasses used in this study proved to be useful and an efficient way of acquiring the biological material needed. However, it also may have induced a significant source of bias in the sampling method. The probability of a badger being picked by a third

party, which included farmers, veterinarians, as well as other members of the public, depended on the destination of the person, their having the time to pick up and deliver the carcass, the possibility of the person being able to stop safely on the road, and of course, investment of the person in the study. Farmers with no cattle were not approached as a source of carcasses, mainly because the engagement of stakeholders was made through the Cheshire TB eradication group; farmers outside this group could have increased the locations of carcasses to areas where there were no cattle. In the same way, the lack of engagement from the Cheshire Council limited our study to roads other than motorways, where stopping is illegal and collection of road-kill is dependant entirely on the council in which the area belongs.

The roads where carcasses were found belonged mainly to class A and class C roads; class A roads tend to be busier and wider roads than B or C roads, which could have been partly the reason why badgers got run over more than in B roads. Residential and unnamed roads (C roads) tend to have less traffic and are generally narrower roads, however, many of these roads, particularly country side roads have high speed limits, scarce or no lighting and low traffic, all of which could have contributed to both a badger being run over and the possibility of someone stopping to pick it up.

Another potential source of bias regarding the location where badger carcasses were found was the fact that where the carcass was found did not necessarily corresponded to where the badger was hit initially, as we heard in several anecdotal accounts, of badgers managing to travel some distance from the site of collision to their point of death. This makes the estimation of the location of setts and crossing pathways more difficult.

In conclusion, this study found that bovine TB is present among badgers in the Cheshire area, and that infection is more widespread than previously thought. It also showed that road kill surveys can be a useful tool to assess disease status and estimated prevalence as well as establish wildlife surveillance schemes. This study suggests a marked increase in bTB in road killed badgers in Cheshire over the space of 25 years, from 0.26% to 21%. However, while this difference could be due to a change in bTB prevalence of this period, this apparent increase could be due to methodological differences, such as might occur due to variation in sampling, collecting and processing carcasses, and laboratory methods. Furthermore, changes in the distribution of badgers and general fluctuation in badger populations within the past 25 years may impact the findings. Unfortunately, other than the proportion of road kill badgers positive for bTB noted above, there is limited information regarding the previous road-killed survey conducted in Cheshire cited by Atkins and

Robinson (2013), so the likely mechanisms underpinning the variation in results from this study's cannot be explained. That badgers and cattle from same areas mostly shared the same *M. bovis* spoligotype, begs the question as to which population drives the expanding edge of the national bTB epidemic. This study was undertaken in 2014, which was by chance a year in which surveillance of bTB in cattle changed and the number, and distribution, of cattle outbreaks increased dramatically. What the spoligotyping of both badger and cattle isolates does suggest is that the Cheshire epidemic is mainly an expansion of that in the counties to the south and east, and not spill over of imported spoligotypes responsible for sporadic cattle outbreaks in Cheshire. Further studies, that might help create a better understanding of 'who infects whom', would benefit from higher resolution typing of isolates than spoligotyping. Whole genome sequencing would be the obvious method to use.

This study also shows the importance and benefits of engaging different types of stakeholders, despite the limitations that individual interests may carry, the wider the network of people involved, the easier it is to overlap those interests and widen the scope and resources of the study. Indeed the outcomes, including lessons learned from this feasibility study, are currently being applied to a national study of TB in badgers in edge counties.

2.2. TB in badgers beyond Cheshire

2.2.1. Introduction

Inevitably, the stakeholder-based study described in 2.1 led to the submission of a small number of badger carcasses from beyond the borders of Cheshire, namely from North Wales, Derbyshire, West Midlands, Shropshire and Staffordshire. In order to maintain stakeholder engagement, these were processed and reported to stakeholders as if part of the study, but not included in the formal Cheshire analysis set out as 2.1. Furthermore, the Cheshire study led to a request from the NFU that a survey be undertaken of road kill badgers in the Stockport area in 2015-16. This section briefly describes and discusses the results of these 'add-on' studies.

As previously outlined, Derbyshire, West Midlands, Shropshire and Staffordshire belong to the HRA, while Stockport, in Greater Manchester, belongs to the LRA, although its south-west border is with Cheshire (an Edge county) (Figure 2.2.1).

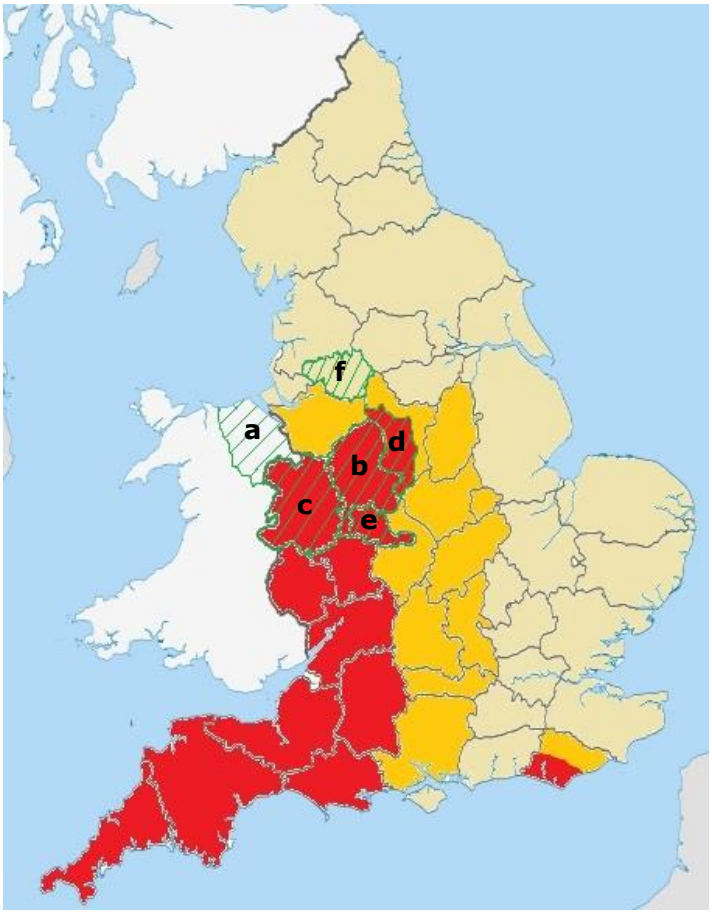

Figure 2.2.1. HRA(red), Edge (orange) and LRA (beige) in England. Areas in green show Clwyd (a), Staffordshire (b), Shropshire(c), Derbyshire (d), West Midlands(e) and Greater Manchester(f).

The Welsh Government does not have a formal categorisation of high and low risk areas, but rather focuses on Intensive Action Areas (IAA) in South Wales that have heightened biosecurity, increased TB testing and (until recently) badger vaccination (APHA(WALES), 2015a). Clwyd in North Wales, on the border with Cheshire and Shropshire border, has a much lower incidence of bTB in cattle than the south-west and south-east areas of Wales, but has seen increasing outbreaks along the border with England. The border breakdowns mainly involve spoligotype 25 (as seen in Shropshire and Cheshire) and Sp9, presumed to result from the importation of cattle from the south (Figure 2.2.2).

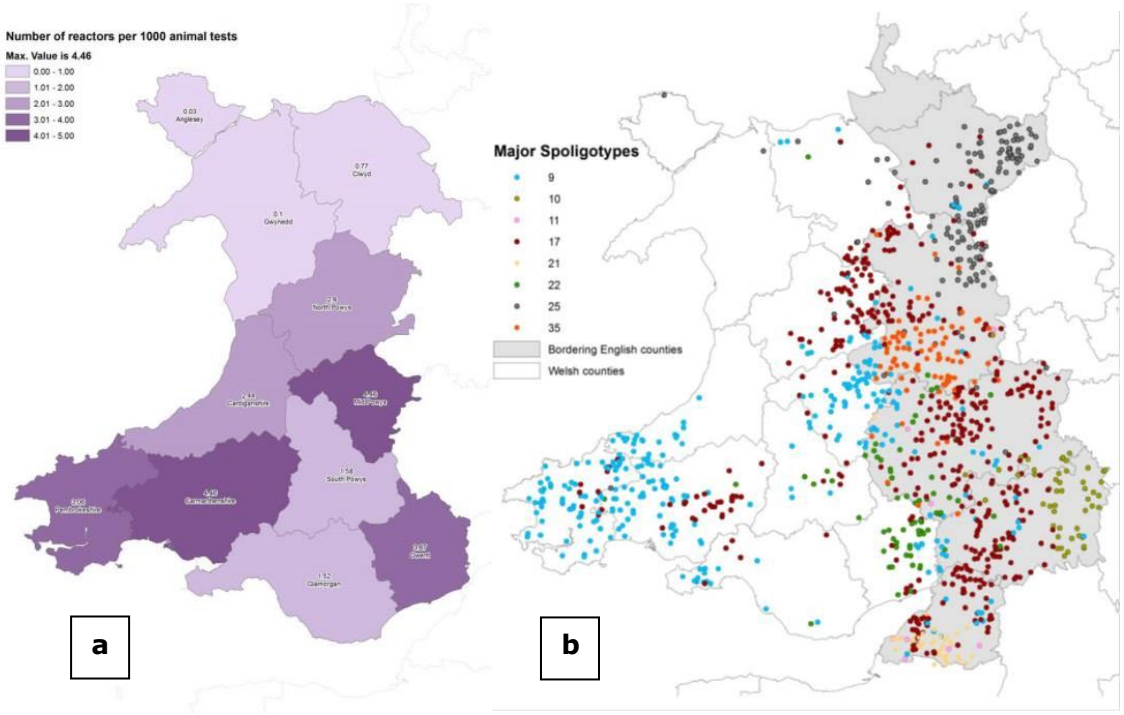

Figure 2.2.2. bTB in Wales (a) and major spoligotypes reported in cattle(b), in 2014 (APHA(WALES), 2015b)

2.2.2. Methods

Badger carcasses and sample collection

Eight badgers were submitted during the 2014 Cheshire study (section 2.1) that came from outside of Cheshire's borders. Five of these were donated by the RSPCA after they were euthanized or died within their wildlife centre at Stapeley Grange.

A total of 30 badgers were submitted by Stockport NFU between September 2015 and June 2016.

All badgers were processed as described in section 2.1.2. Briefly, carcasses delivered to the University of Liverpool Veterinary School were kept at 4°C for no more than 24 hours after which they were transported into the CL3 laboratory for post mortem examination. Collected tissues included: any visible lesions compatible with TB, superficial, thoracic and abdominal lymph nodes as well as lung lobes, spleen, liver and kidneys. Organs and lymph nodes were processed into 'pools' (lung, abdominal, thoracic, carcass and head and neck). Tissues with visible lesions were stained using modified Ziehl-Neelsen method (Engelkirk and Duben-Engelkirk, 2008). Individual tissue samples were stored at -80°C for further study.

Attempted isolation of *M. bovis*

Badger carcasses and tissue samples were processed as previously described in section 2.1.2 and cultures were incubated in both Stonebrink Selective agar (BD Diagnostics, Oxford) and Lowenstein-Jensen with pyruvate slopes (Media for Mycobacteria Ltd. Penarth) and incubated at 37°C for a minimum 12 weeks (Cousins, 2012).

Characterisation of *M. bovis* isolates from badgers

DNA was extracted from colonies by heating a suspension of 1-2 colonies in 100 ml of DNA-free water at 80°C for 30 minutes. Spoligotyping was (or at the time of writing, still is being) carried out at APHA Weybridge and Nottingham University by Ben Swift using a DNA microarray technology (Kamerbeek et al., 1997, Ruettger et al., 2012).

Cattle data

At the time of the study, no formal cattle data were available for analysis, beyond those mentioned in the introduction.

Analytical and Statistical approaches

Estimated prevalence was calculated from the sampled badgers as well as likelihood of bTB status based on age, sex and season. These analyses were performed using chi square tests when the expected frequencies were 5 or more for each cell. When more than 20% of the data had expected frequencies of 5 or less, Fisher's exact probability test was performed, using the Freeman-Halton's extension when the variable consisted of more than 2 categories. Analysis was done using the Vassar stats website for statistical computation (http://vassarstats.net/).

Kulldorf's spatial scan statistic (SaTScan™ ver. 9.4.2) was used to investigate the presence of clusters of bTB- positive badgers in Stockport (Kulldorff and Nagarwalla, 1995, Kulldorff, 1997, Kulldorff et al., 2005).

The location of badger carcasses and cattle holdings was mapped using the "Rgoolgemaps" package (Loecher, M., 2014) in R version 3.3.1 (R Core Team, 2014), with locations jittered for presentation as it was agreed with the stakeholders that precise locations would not be disclosed for reasons of confidentiality.

2.2.3. Results

A total of 38 badgers were examined for bTB. Locations of the non-Stockport badgers included: West Midlands, North Wales, Derbyshire, Staffordshire and Shropshire. The location of the badger carcasses is shown in Figure 2.2.3.

Two adult male carcasses, one from Shropshire and one from Stockport, had dental wear and body mass suggestive of being over 3 years old. One of these carcasses, brought from Shropshire, also had characteristic tuberculous lesions disseminated in lungs and other organs. One more carcass, from North Wales had a small lesion limited to an axillary lymph node.

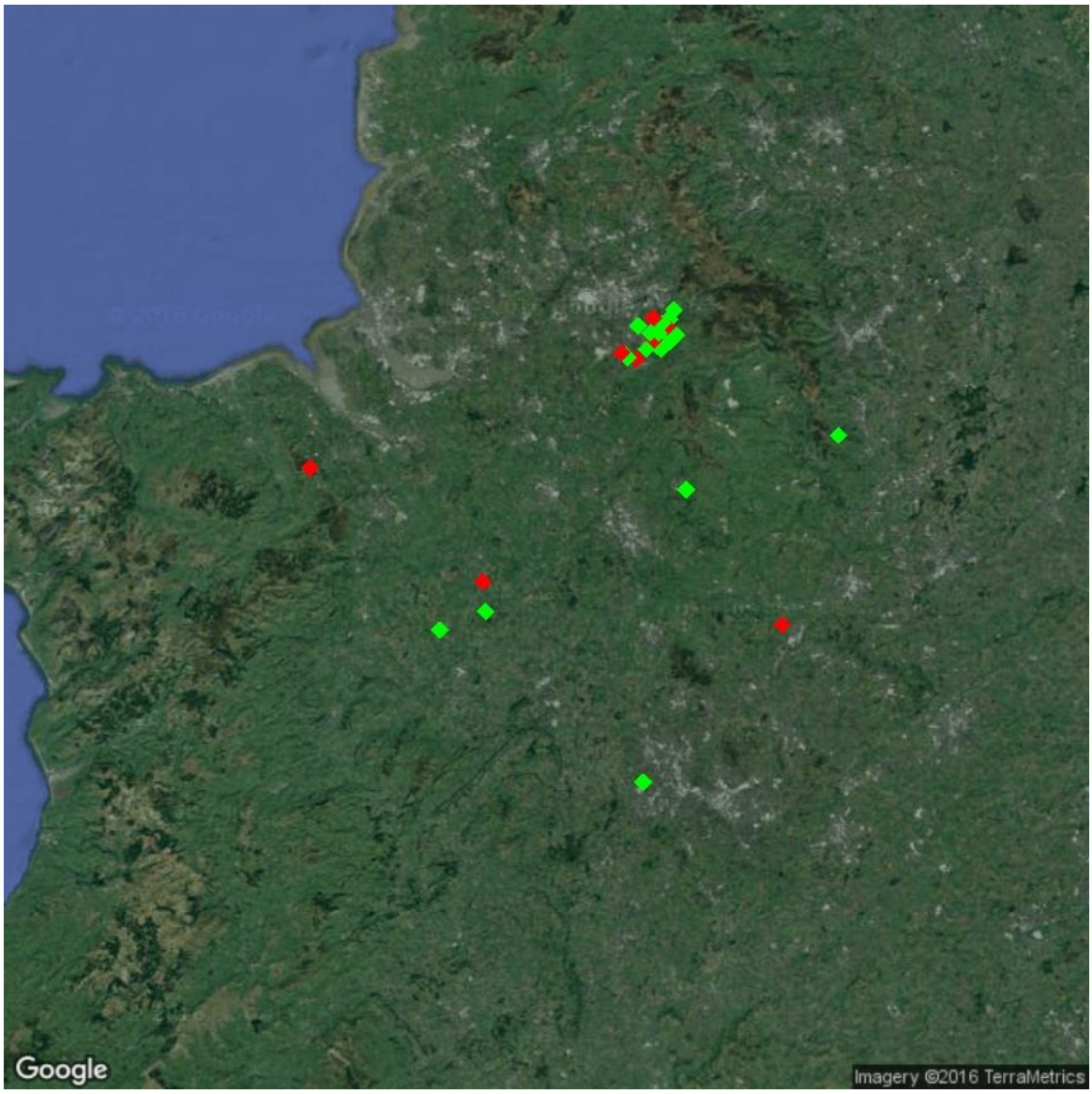

Figure 2.2.3. Jittered locations of bTB positive (red) and negative (green) badger carcasses which were not included in section 2.1

Three of the eight badgers submitted from outside of Cheshire and Stockport were positive for TB, however, only two were *M. bovis,* SP25 while the isolate from a badger from North Wales was *Mycobacterium microti* (SP34).

Stockport badgers

Of the 30 badger carcasses from Stockport, all were adults. Four badgers had male pseudohermaphroditism characteristics at post mortem examination (Bigliardi et al., 2011). If the pseudohermaphrodites are counted as males, then 50% of the Stockport badgers were males. *M. bovis* was isolated from eight of these (26.7%, 95% CI 14.2 to 44.5) and a small lesion limited to one axillary lymph node was found. At the time of writing, spoligotyping of these isolates is still underway. The jittered locations of the carcasses are shown in Figure 2.2.4.

Figure 2.2.4. Locations of bTB positive (red) and negative (white) badger carcasses from the Stockport area.

Infection with bTB was not obviously influenced by sex as 4 males and 4 females were bTB positive (p=1) or seasonality (p=0.6). Carcass submissions did appear to vary seasonally, with a peak in autumn (47%) although most infected carcasses were submitted in spring (62.5%) (Table 2.2.1, Fig. 2.2.5). No clusters of infected badgers were detected using Kulldorff's spatial scan statistic.

Table 2.2.1. Prevalence, confidence intervals and statistical tests (X^2, *Freeman-Halton's extension of the Fisher's exact probability test) results according to age, sex and season categories for Stockport-only badgers.

	Positive	Negative	Prevalence	95% Confidence limits Lower	Upper	X^2	p
Juvenile	0	0					
Adult	8	22	26.7%	14.2	44.5		
Males	4	11	26.7	10.9	51.9	0	1
Females	4	11	26.7	10.9	51.9		
Winter	1	6	14.3	2.6	51.3		
Spring	5	3	62.5	30.6	86.3		0.08*
Summer	0	1					
Autumn	2	12	14.3	4.0	39.9		

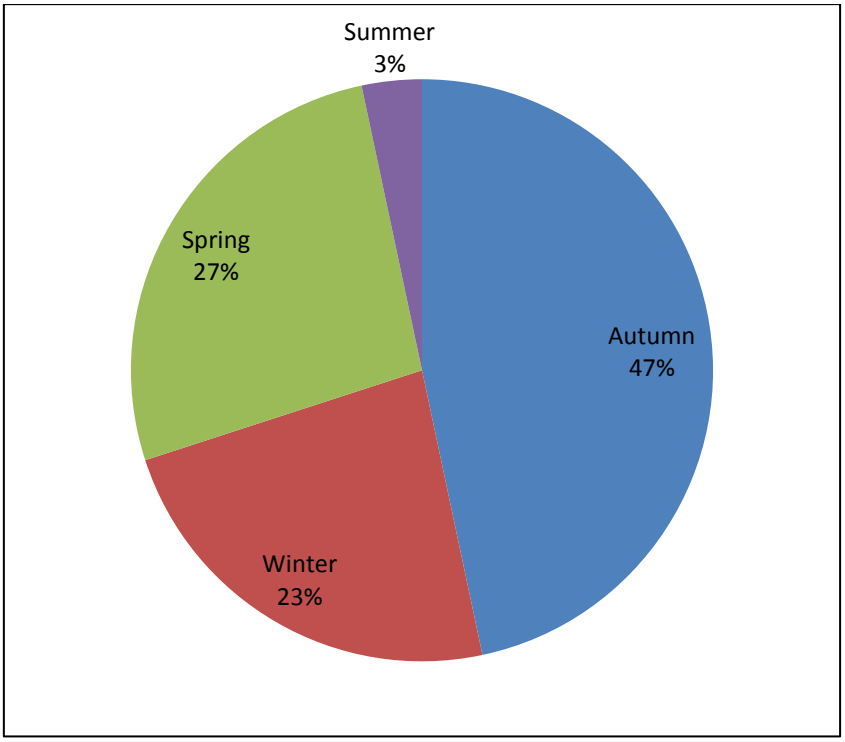

Figure 2.2.5. Badger carcasses from Stockport submitted to the study by season.

2.2.4. Discussion

Most of the carcasses examined in this section were from Stockport, and will be discussed as a stand-alone project.

It is not surprising that the isolates from Shropshire and Staffordshire were *M. bovis* SP25, since these areas are in the HRA and SP25 is the regional spoligotype. Perhaps more interesting was the isolation from a badger in North Wales of *M. microti,* spoligotype 34. *M. microti*, like all members of the MTB Complex, has a wide host range that includes several rodents, camelids, cats, squirrel monkeys, meerkats, pigs, wild boars, dogs, cattle and humans (Huitema and Jaartsveld, 1967, Cavanagh et al., 2002, Deforges et al., 2004, Jahans et al., 2004, Oevermann et al., 2004, Taylor et al., 2006, Henrich et al., 2007, Gunn-Moore et al., 2011, Palgrave et al., 2012). Its main host, from which it takes both its scientific name and common name (vole TB) is field voles (*Microtus agrestis*). It has been studied particularly in vole populations in Northumbria/Cumbria (Cavanagh et al., 2002) but was first described and studied in voles in North Wales (Wells, 1937, Chitty, 1954). Infection of cattle – or at least isolation of *M. microti* from cattle, is rare and has not previously been described in Wales (APHA(WALES), 2015b). The isolation of *M. microti* from one badger must not be over-interpreted, however it is worth noting that North Wales has a historically low prevalence of bTB in cattle, and Smith et al. (2009) suggested that natural infection with *M. microti* might give population-level protection of wildlife against infection with *M. bovis* and so explain the low prevalence of *M. bovis* in cattle in those areas where *M. microti* circulates. As a member of the MTB Complex, *M. microti* is antigenically very similar to *M. bovis*, and was in the past seriously considered as an alternative to BCG for immunisation against tuberculosis in people (Wells, 1949, Wells and Wylie, 1954, Paul, 1961, Hart and Sutherland, 1977).

The estimated prevalence of bTB in Stockport was 26.7% (although with wide confidence intervals), which, for an area that has low TB incidence in cattle, is surprisingly high. It will be interesting to see the results of spoligotyping. No cattle location data were made available, but several badgers came from areas near holdings that had just had their OTF status suspended or withdrawn (Pers. Comm.). As in the Cheshire study, any co-location of badger and cattle cases does not prove cross-species transmission nor allow inferences to be made about the direction of any transmission. As in the Cheshire study, whole genome sequencing of badger and cattle isolates may provide a better understanding of any such transmission.

The Stockport study had a very relatively small sample size, and as with most areas in the North of England, the population of badgers around Stockport is unknown. Still, these results do demonstrate the importance of including areas which are farther away from the formal edge region in future studies. Stockport is considered LRA, even though it is very close to the Edge area, and the study of badgers with bTB in LRAs could shed new light into the dynamics of the bTB epidemic in both cattle and badgers.

As in section 2.1, there was no significant influence on TB infection of sex, age or season in found dead badger carcasses. However, the seasons with most badger carcasses submitted were again spring and autumn, reinforcing the importance of these times of year for future studies.

No cluster of infection in badgers was found, and the distribution bTB infected badgers in Stockport seems was fairly diffuse and not limited to, for example, the Cheshire border. Furthermore, not all badgers were found in the countryside or near farms: two badgers, one positive and one negative, were found in suburban areas. This may be significant in terms of recent reports of bTB in domestic cats, which in some cases transmitted infections to their owners (Gunn-Moore, 2014).

A coincidental but interesting finding of this study and the Cheshire study (section 2.1) is the high rate of pseudohermaphrodite badgers found, particularly in Stockport. As far as we know, this is the first time this condition has been reported in European badgers. Although both populations studied were small, rates of pseudhermaphroditism of 2% and 11% are both high, and particularly so in the Stockport population. It is tempting to suggest the presence of pollutants in the environment that affect sexual development of these animals, particularly in the more urban Stockport area when compared to Cheshire. Future studies might look for evidence of endocrine disruptors in the tissues from badgers: and if found, badgers (which eat large numbers of earthworms and so might be exposed to high levels of soil contaminants) might even be sentinels for terrestrial pollution.

Limitations of the study

As with the study described in section 2.1, the collection of carcasses relied upon members of a stakeholder network, however, in the case of the Stockport, all carcasses were collected through the Local Authority. It is not clear how this might bias sample collection compared to collection by farmers and badger groups.

Some of the biases discussed in the previous study apply to that presented in this chapter, for example, basing sample collection to road traffic accidents reduces the possibility of investigating the bTB status in badgers in areas with no road access. However, the areas covered, and especially Stockport which is quite urban, have a relatively high road density.

The main limitation to the Stockport study was the sample size, which was determined by the resources available at the time. A larger sample size might have enabled greater confidence in the prevalence estimate. On the other hand, the overall density of badgers in the area (and thus the proportion tested, is unknown. Nonetheless, this study showed bTB to be present in badgers in a LRA for cattle, which was not expected.

Few conclusions can be drawn from those badgers collected outside the Cheshire or Stockport area, beyond that the expected spoligotype was found south of Cheshire, and that finding *M microti* in a badger from N Wales supports the conclusion from the Stockport study that a larger investigation of badgers beyond the edge of the cattle epidemic might provide useful epidemiological information.

In conclusion, this study showed a high estimated prevalence of bTB positive badgers found in an area considered of low risk. More extensive studies of badgers in the LR areas are required to better understand the relationship between bTB in cattle and wildlife. The presence of *M. microti* in badgers and the correlation with low incidence of *M. bovis* in cattle inhabiting the same area is another path worth exploring.

3. *Cryptosporidium* and *Giardia* in the environment

3.1. Detection of *Cryptosporidium* and *Giardia* in wild rodents, livestock and water in Llyn Cowlyd

3.1.1. Introduction

Cryptosporidium and *Giardia* are responsible for, respectively, more than 8 and 28 million cases of diarrhoeal disease in humans worldwide (WHO, 2015), most of which are associated with contaminated water. The economic cost of dealing with outbreaks of water-associated diseases, cryptosporidiosis and giardiasis included, makes water treatment highly important. In the USA it is estimated that $970 million are spent every year on managing waterborne disease outbreaks: one outbreak of cryptosporidiosis in Milwaukee in 1993 involved 400,000 cases and cost around $96.2 million (Corso et al., 2003, Collier et al., 2012). More recently, in 2015, an outbreak of cryptosporidiosis in Lancashire affected 300,000 home owners and cost United Utilities over £25 million in compensation alone (BBC, 2015, Wallace, 2015).

Infective cysts of both parasites can be found in the environment, including soil, water and faeces, and can survive for long periods. *Cryptosporidium* oocysts can survive in water and soil for more than 12 weeks, even if temperatures are as low as -4°C, while *Giardia* cysts can remain infective up to 11 weeks in water at 4°C (Olson et al., 1999, Robertson and Gjerde, 2006). This resilience presents challenges for water utilities worldwide. Drinking water supplies can be readily contaminated through run-off from agricultural land, wildlife or by contamination with human sewage (Lechevallier et al., 1991, Smith et al., 1995). Moreover, both protozoa have relatively low infectious doses to humans: as few as 10 oocysts for *Cryptosporidium parvum* (Okhuysen et al., 1999) and 10-100 cysts of *Giardia duodenalis* (Rendtorff, 1954) can cause infection and disease in healthy adults.

Conventional water treatment is based on the use of multiple barriers to stop contaminants reaching homes, usually consisting of coagulation, flocculation, sedimentation and filtration, which physically remove most protozoa, followed by disinfection (Betancourt and Rose, 2004). These methods are adequate for reducing *Cryptosporidium* and *Giardia* (oo)cysts in the water, however their effectiveness depends on the original concentration of (oo)cysts, which, in the case of *Cryptosporidium* it can be over 5000 oocysts per litre in surface waters (Rose, 1997).

Disinfection, the last stage of conventional water treatments, is most commonly through treatment with chlorine, which is effective at killing *Giardia* cysts at high concentrations and long exposure times but not *Cryptosporidium* oocysts. Ozone, chlorine dioxide and UV light are the only methods currently accepted to provide adequate inactivation of *Cryptosporidium* oocysts (Korich et al., 1990, Betancourt and Rose, 2004). Thus a great deal of effort is put into preventing *Cryptosporidium* and *Giardia* from entering the water supply.

Llyn Cowlyd, located in the Carneddau Mountains in North Wales, is the largest water reservoir supplying the Bryn Cowlyd Water Works, which in turn provides drinking water to over 44,000 homes and businesses in Llandudno, Llanfairfechan, Llanrwst, Cowlyn Bay and Conwy Valley (Figure 3.1.1). There are no recreational activities on the reservoir, although multiple footpaths / biking trails run alongside the water. Furthermore, both cattle and sheep are grazed around the reservoir, although largely fenced away from direct contact with the lake itself.

Over the last eight years an increased concentration of *Cryptosporidium* oocysts has been detected in the raw water extracted from the reservoir during the summer months, and this prompted the question as to what the origin of these cysts as well as their human pathogenic potential might be. Bryn Cowlyd Water works uses UV light to treat the water prior to public distribution, and there have been no outbreaks in people associated with water from the reservoir. However Welsh Water / Dwr Cymru (DCWW) was understandably concerned to determine the source of contamination and the risks associated with it in order to mitigate any potential future public health issues.

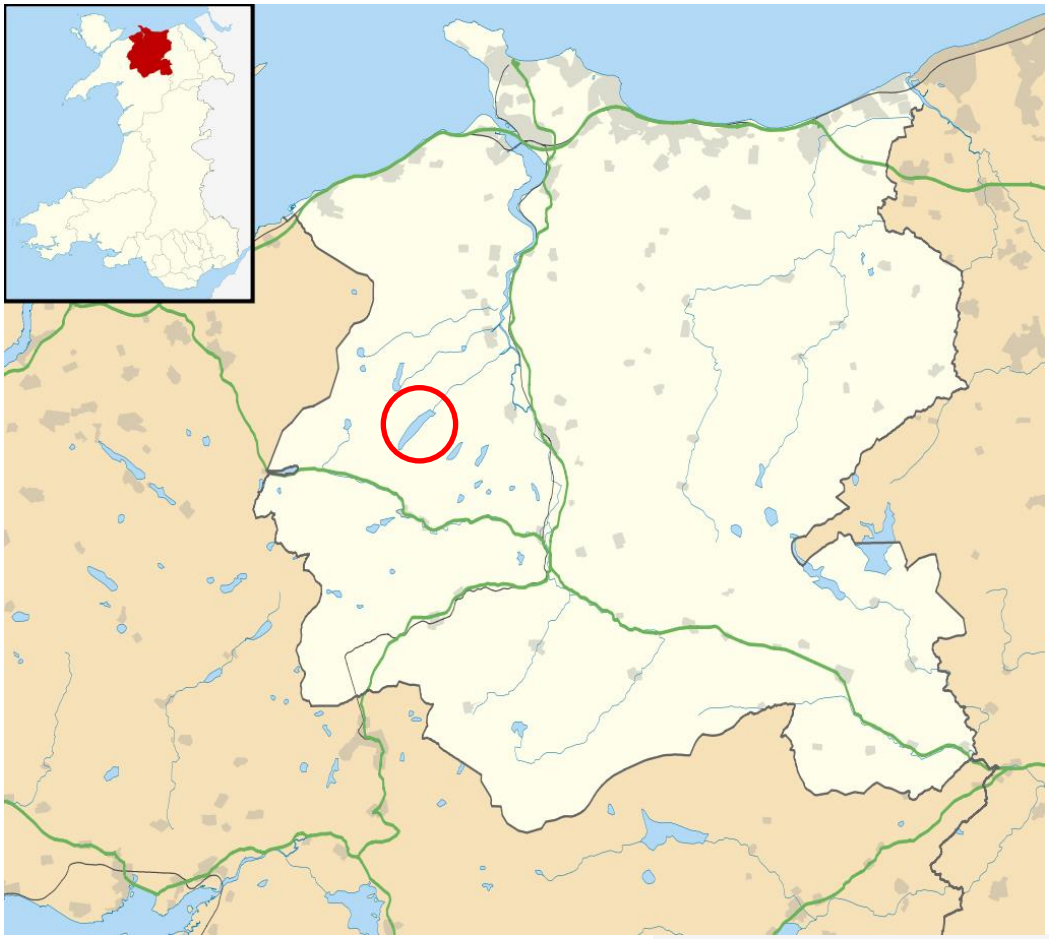

Figure 3.1.1. Location of the Conwy Valley and Llyn Cowlyd (red) Contains Ordnance Survey data © Crown copyright and database right.

The study

A project to understand the origin(s) and, by genotyping, the pathogenic potential of the *Cryptosporidium* oocysts found in Llyn Cowlyd was developed jointly with Welsh Water and the Public Health Wales Cryptosporidium Reference Unit (CRU, Swansea). The aim of this study was to identify the possible wildlife and livestock sources for *Cryptosporidium* oocysts, and compare them with any cryptosporidia found in water samples taken from the reservoir and its feeder streams during the same period. As the samples generated could also be used to investigate potential *Giardia* contamination, it was agreed also to add the detection and characterisation of *Giardia* to the study.

3.1.2. Methods

Trapping and sample collection

In order to determine a sampling regimen, the site was visited in 2014 to establish both the geography of the site and what species might be present that could be contaminating the water of the reservoir. Following finding signs of field voles (*Microtus agrestis*) and wood mice (*Apodemius sylvaticus)*, a feasibility/pilot study was undertaken to determine the rodent species present and trial trapping approaches. Ethics approval was granted by the appropriate panels and committees from the University of Liverpool and the University of Nottingham (VREC267, SVMV 1747 160425, SVMV 1462 150515).

The main study took part in the summer of 2015, with the aim of straddling the time of year (July-August) when the peak in water contamination with *Cryptosporidium* had been previously found by Welsh Water /Dwr Cymru

Rodent sampling

A live trap and release scheme using Longworth traps (Figure 3.1.2) was used to capture wild rodents in the surroundings of Llyn Cowlyd for nine weeks beginning on the 15th of June, 2015, using the sampling schedule showed in Table 3.1.1

Figure 3.1.2. Longworth trap for live trapping rodents. The door at the entrance of the tunnel automatically closes when a lever at the end is pressed by the rodent as it walks past it to the main chamber where bedding and food was allocated.

Fifty traps were deployed in forty by forty metre grids, with two grids sampled every week (Appendix A). The locations of the grids were selected according to evidence of rodent activity and to ensure different habitat types were included along the edge of the lake. The approximate location of the grids is shown in Figure 3.1.3. Traps were filled with hay as bedding and mixed grains for food, and autoclaved to ensure no contamination of samples occurred.

Table 3.1.1. Starting dates and grids selected for each of the nine weeks of the trial.

Week	Starting Date	Grid
1	15th of June	1 and 2
2	22nd of June	3 and 4
3	29th of June	5 and 6
4	20th of July	1 and 2
5	27th of July	3 and 4
6	3rd of August	5 and 6
7	1st of September	1 and 2
8	7th of September	5 and 6
9	14th of September	3 and 4

The traps were set on Monday morning, and left overnight. Fresh carrot or apple pieces were added to provide a source of water to any animal trapped. Tuesday to Thursdays traps were checked for any trapped rodents. Traps with rodents inside were emptied into transparent bags, and the site, sex, age (adult/juvenile) and the species recorded. All trapped rodents were marked by trimming fur from their backs in order to recognise recaptured animals (Figure 3.1.4). All rodents were then released.

Faecal samples from the traps were collected in sterile pots with location, grid and trap number recorded (Figure 3.1.5). These traps were then emptied, cleaned and swabbed with ethanol and sterile bedding and seeds were put inside and the traps were set again.

On Thursdays all traps were taken back to the Leahurst campus for cleaning and autoclaving prior to use at another site. The specifics of the trapping scheme are described in Appendix A.

Figure 3.1.6 shows the landscape of the lake, as well as livestock observed near the shore and the heavy rainfall experienced during the study.

Figure 3.1.3. Distribution and location of the grids (Red squares) and the feeder streams sample sites (blue circles). Each grid measures 50x50 metres. Image from Google Earth©

Figure 3.1.4. Traps with rodents were emptied in transparent bags and the rodents retrieved for sexing, aging, species identification and marking. All rodents were released afterwards.

Figure 3.1.5. Faecal samples were collected from the traps and transported back to the laboratory in sterile containers.

Figure 3.1.6 From the top, Llyn Cowlyd from the far end of the reservoir, sheep and cattle present near the shore of the reservoir. Heavy rain observed during the study.

Sheep and cattle sample collection

Samples comprising approximately 50 grams of fresh sheep and cattle faeces found within the same grids were collected in disposable plastic bags with location, date and species recorded. They were transported back to the laboratory and kept at 4°C until processing.

Water samples

Samples from feeder streams were collected every week by Dr Keith Osborn using a portable sampling rig fitted with an Idexx Filta-Max filter. Samples were processed by DCWW using immunomagentic separation (IMS) beads and forwarded to the PHW Cryptosporidium Reference Unit (CRU, Swansea) for further analysis.

Additionally, water entering the Bryn Cowlyd Water Treatment Works was sampled between 12th January and 19th November and examined for *Cryptosporidium* by the DCWW staff (Glaslyn, Newport) as part of their normal water testing protocols using *Cryptosporidium* specific IMS beads. Positive samples collected between 3rd June and 28th September, were forwarded to the PHW CRU (Swansea) for genotyping.

Laboratory methods

Rodent DNA extraction (QIAGEN)

DNA was extracted from rodent faecal samples using the QIAmp® DNA fast stool mini kit following the manufacturer's (Qiagen's) instructions. A summary of the method can be seen in Appendix B.

DNA extraction from cattle and sheep samples

Concentration of oocysts from cattle and sheep samples

Oocysts were concentrated from livestock faeces using a method developed by Beth Wells and collaborators at the Moredun Institute (Scotland) (personal communication). Briefly, 50g of faeces, 700ml of water and 7ml 2% sulphuric acid were added to a 1000 ml glass column. The mixture was stirred using a magnetic stirrer for 5 minutes and then left to settle for one hour to remove gross organic material. The clear layer (containing the oocysts) was removed and transferred to 250ml bottles which were then centrifuged at 1000xg for 20 minutes. The resultant pellets were

resuspended in 3ml water and centrifuged again at 3000 xg for 5 minutes. Each pellet was retrieved and resuspended in 8ml saturated salt solution with 3ml of distilled water carefully added to the surface before centrifugation at 1100 xg for eight minutes. The specific density of the oocysts means they float towards the middle layer between the saturated salt and the distilled water.

Using a pipette, and paying special attention not to disturb the layers, the top water layer was swirled until it became cloudy, due to the presence of oocysts, and was removed and mixed with distilled water in a Falcon tube to reach a final volume of 10ml. This suspension was centrifuged at 1100 xg for 5 minutes and the supernatant was discarded. The pellet was re suspended in 1ml of TE buffer.

DNA extraction

DNA was extracted using a DNA, RNA and protein purification kit (NucleoSpin® Tissue, Macherey-Nagel) following the modifications from Wells et al. (2015b). The full protocol can be found in Appendix B.

Cryptosporidium spp. and *Giardia spp*. detection and characterisation*:*

Characterisation of *Cryptosporidium* and *Giardia* differed according to sample type and target organism. New real-time PCR assays developed as part of the Aquavalens project (www.aquavalens.org) were used to detect *Cryptosporidium* and *Giardia* DNA, briefly, for *Cryptosporidium*, DNA from all sample types (feeder streams, animal faeces and raw water) was first subjected to amplification using the real-time PCR "ALC2" assay targeting the SSU rRNA gene to determine presence of *Cryptosporidium* DNA (Chalmers, 2015) using the primers ALC2F (AAGTATAAACCCCTTTACAAGTATCAA), ALC2R (TATTATTCCATGCTGGAGTATTCAAG) and ALC2T (FAM-ACTTTGAGAAAATTAGAGTGCTT-MGB-NFQ).

Where *Cryptosporidium* was not confirmed by this method, DNA was further amplified using a conventional nested SSU rDNA approach following conditions specified by Jiang et al. (2005) and primers 18s1F (TTCTAGAGCTAATACATGCG), 18s1R (CCCATTTCCTTCGAAACAGGA), 18s2F (GGAAGGGTTGTATTTATTAGATAAAG), 18s2R (AAGGAGTAAGGAACAACCTCCA) and 18SJR2 (CTCATAAGGTGCTGAAGGAGTA), followed by sequencing of amplicons. Real–time PCR and amplicon sequencing for both microorganisms were conducted in the *Cryptosporidium* Reference Unit (CRU, Swansea) by the author and Dr. Kristin Elwin.

Additionally, all DNA samples were screened by Dr. Kristin Elwin for the presence of *C. parvum*, using a highly specific and sensitive quantitative real-time PCR (qPCR) targeting a *C. parvum*-specific region of the Lib13 locus. The real-time PCRs used a commercial mastermix and conditions specified by Hadfield et al. (2011). Primers used were CRULib13F (TCCTTGAAATGAATATTTGTGACTCG), CRULib13RCp (TTAATGTGGTAGTTGCGGTTGAAC) and CRULib13TMCp (VIC TATCTCTTCGTAGCGGCGTA MGB-NFQ). Samples containing *C. parvum* were further investigated by PCR-sequencing of part of the GP60 gene to establish the genotype, using a cocktail of primers to accommodation likely variation at this locus (Chalmers et al., 2016).

Giardia detection and characterisation was carried out on DNA from animal and feeder stream samples using real-time PCR amplification of a region of the β-giardin gene following the conditions specified by Chalmers et al. (2015). The primers used were BGF2 (GAGGCCCTCAAGAGCCTGAA), BGF2R2 (ACACTCGACGAGCTTCGTGTT) and BG2T (VIC-ATCGAGAAGGAGACGATCGC-MGB-NFQ).
Cryptosporidium amplicons were purified using a QIAquick PCR purification kit (QAGEN) following manufacturers instruction and sequenced by Source Bioscience. Sequences analysis was performed using Chromas Pro1.4 (Technelysium Pty. Ltd) by editing and assembling nucleotide sequences and compared against all sequences on Genbank using the Basic Local Alignment Sequence Tool (BLAST).

3.1.3. Results

Wild rodents

A total of 77 rodents and one insectivore were sampled between the months of June and September 2015. Four species were sampled, bank vole (*Myodes glareolus*), field vole (*Microtus agrestis*), woodmouse (*Apodemus sylvaticus*) and a common shrew (*Sorex araneus*) (Figure 3.1.7). Field voles represented the majority of the trapped animals (42%) followed by woodmice (36%).

In addition, twenty samples were from unknown animals as small mammal faecal droppings were found inside the traps but no animal was found.

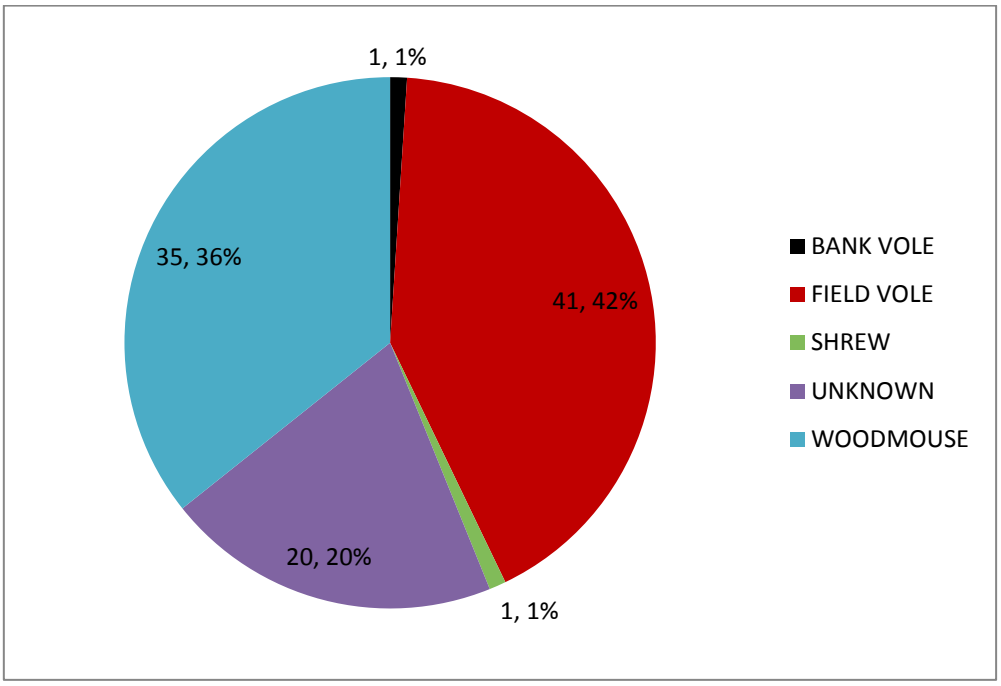

Figure 3.1.7. Proportion of wild animals trapped in Llyn Cowlyd during June-September 2015

Overall, 42% of the identified trapped animals were males and 58% females, the age and sex of the shrew could not be determined. Almost all rodents (91%) were adults: 90% and 91% of field voles and wood mice respectively. Based on size and pelage, 9% of the identified rodents were considered juveniles. The beginning of summer (June) was the month with less trapping success (6%) while mid - summer (July-August) and early autumn (September) yielded 94% of the total trapping with 47% for each time period (Table 3.1.2). The grids with most sampled animals were 1, 2 and 3, which provided 23%, 20% and 18% respectively of trapped animals.

Cryptosporidium spp. and *Giardia spp.* findings

Wild rodents

Of the sampled wild rodents, 35 were positive for *Cryptosporidium spp*. (35.7% CI 95% 26.9-45.6%) and 11 were positive for *Giardia spp.* (11.2% CI 95% 6.4-19%). In four cases animals presented a *Giardia* and *Cryptosporidium* co-infection (Table 3.1.2). *Giardia* was only detected in field vole samples. Of the positive samples for *Cryptosporidium, 63%* were novel genotypes (A), while only 5.71% were of the known zoonotic species *C. parvum*. Overall, 37% of amplicon sequences matched previously described sequences and 63% were novel, all of which were highly similar to each other but distinct from known species sequences.

Livestock

Eleven livestock faecal samples from sheep (10) and cattle (1) were collected for analysis. A total of four samples were positive for *Cryptosporidium* (36.4% CI 95% 15.2-64.6%) three from sheep and one from cattle. Sequences from sheep positive samples were determined as *C. parvum* (2) and C*. bovis* (1). One cattle sample was positive for *C. ryanae*. The two sheep samples containing *C. parvum* were also positive for *Giardia spp.* (20% CI 95% 5.7-51%) (Table 3.1.2).

Water samples

Feeder streams

A total of 72 samples from feeder streams were tested: 19 were positive for *Cryptosporidium* (26.4% CI 95% 17.6-37.6%) and 37 were positive for *Giardia* (51.4% CI 95% 40.1-62.6%). *C. ubiquitum* was found in eleven samples (58%), three of which were mixed with a novel genotype. This same novel genotype was found in other five feeder stream samples (26%). One sample contained a different novel genotype and a further sample contained the novel genotype found in rodent samples (Table 3.1.2).

Raw water

A total of 84 raw water samples were taken, however, due to a contamination problem, only 49 were analysed: 22 were positive for *Cryptosporidium* (44.9% CI 95% 31.9-58.7%). One sample was positive for *C. parvum* (4.6%), and another five were positive for *C. ubiquitum,* three of which were mixed with a novel genotype (D) and one with the same genotype as that found in feeder streams

(B). The novel genotype (D) was also found in 14 further samples. One more genotype (E), was also found in one sample. One sample contained the same novel genotype found in rodent samples (A).

A breakdown of *Cryptosporidium* species and genotypes by source of isolation obtained during this study is shown in Figure 3.1.8. Raw water samples were not subjected to *Giardia* analysis.

A total of 51 samples were taken by DCWW as part of their weekly water inspection. *Cryptosporidium* oocysts were observed in 25 samples with the highest counts between 5th and 8th of August (data not shown).

Table 3.1.2. Total samples and *Cryptosporidium* species and sequence types as well as *Giardia* species for each positive sample.

Month	Species/ sample	Total sampled	*Cryptosporidium spp.* +	*Cryptosporidium* species/ sequence type	*Giardia spp.*
June	bank vole	1	0		0
	field vole	4	3	Muskrat gt2(2), novel A(1)	0
	shrew	0	0		0
	unknown	0	0		0
	woodmouse	1	0		0
	sheep	3	2	*C. parvum* (2)	2
	feeder streams	18	0		9
	raw water	20	11	*C. ubiquitum* + novel D (1), novel D (8), novel B(1), novel E (1)	Not tested for *Giardia.*
July-August	bank vole	0	0		0
	field vole	23	8	UKE7(2), muskrat gt2(3), novel A(3)	7
	shrew	1	0		0
	unknown	3	1	Muskrat gt2(1)	0
	woodmouse	19	2	*C. ubiquitum*(1), novel A (1)	0
	sheep	3	0		0
	feeder streams	40	13	*C. ubiquitum*(5), *C. ubiquitum* + novel C(2), novel B(6)	16
	raw water	23	10	*C. ubiquitum* + novel D(2), *C. ubiquitum* + novel A(1), novel D(5), *C. ubiquitum*(1), novel E(1)	Not tested for *Giardia.*
Sept.	bank vole	0	0		0
	field vole	14	11	Novel A(9), UKE1(2)	4
	shrew	0	0		0
	unknown	17	2	Novel A(2)	0
	woodmouse	15	8	Novel A(6), *C. parvum*(2)	0
	sheep	4	1	*C. bovis*	0
	cattle	1	1	*C. ryanae*	0
	feeder streams	14	6	*C. ubiquitum*(3), *C. ubiquitum* + novel C(1), novel B(2)	12
	raw water	6	1	*C. parvum*	Not tested for *Giardia.*

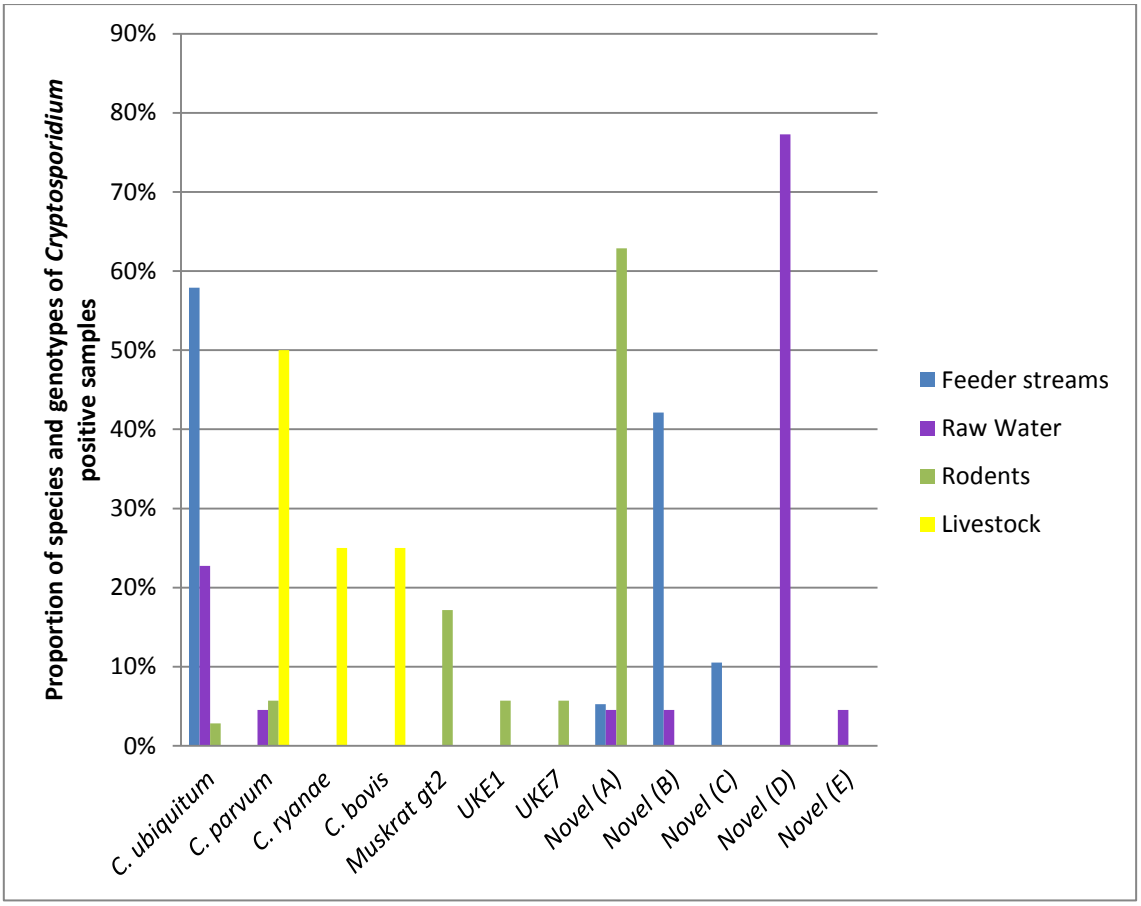

Figure 3.1.8. Proportion of *Cryptosporidium* species and genotypes from *Cryptosporidium* positive samples from all sources.

3.1.4. Discussion

In 2012, the United Kingdom had the highest number of confirmed cases of cryptosporidiasis and the second highest number of cases of giardiasis in the EU, with over 6000 and 4000 cases respectively (ECDC, 2014). If under reporting is taken into account, (Tam et al., 2012) the actual numbers of cases might be over 40,000 and 50,000 respectively. Most infections occur through ingestion of water or food contaminated with infective (oo)cysts, which can be found on surface waters that are intended for human consumption. These highly resilient (oo)cysts are resistant to most water disinfection treatments, making UV light irradiation the method of choice to eliminate them from the water, however, the efficacy of this treatment depends on the original burden of cysts in the water. Determining the source of these (oo)cysts can aid in controlling the amount that can find their way into the water systems.

The aim of this study was to determine the role of animals, particularly wildlife, as sources of *Cryptosporidium* and *Giardia* (oo)cysts found at the Llyn Cowlyd water works, as well as the zoonotic potential of any strains or species found. For several years, a summer annual peak in *Cryptosporidium* oocysts had been observed, hence this study focussed on the beginning of summer (June) to the beginning of autumn (September) 2015.

We found little evidence of wildlife diversity in the Llyn Cowlyd area. A pilot study in 2014 showed that the species most likely to be found were woodmouse (*Apodemus sylvaticus*), bank vole (*Myodes glareolus*), field vole (*Microtus agrestis*) and common shrew (*Sorex araneus*). Small fish and amphibians were observed every now and then, and the lake is home to Arctic char (a relic population from the ice age) and small numbers of brown trout (Welsh Water / Dwr Cymru – pers communication). Relatively small numbers of wild birds, mainly small passerines (in particular wheatears, *Oenanthe oenanthe* and meadow pipits, *Anthus pratensis*) and at least one pair of ravens were observed regularly. However, the landscape was not suitable for trapping birds or finding fresh droppings, and therefore no birds were sampled. Additionally, no water fowl species were observed on the reservoir. Bird watching websites for Snowdonia tend to ignore Llyn Cowlyd as, unlike other lakes in the area, it has few birds of interest to ornithologists.

The prevalence of *Cryptosporidium spp.* in wild rodents in this study was 36%. This is similar to that reported in some other studies in different species of wild rodents (Klesius et al., 1986, Chalmers et al., 1997, Chilvers et al., 1998, Sturdee et al., 1999, Kilonzo et al., 2013, Song et al., 2015), but higher

than that reported in others (Laakkonen et al., 1994, Hikosaka and Nakai, 2005, Foo et al., 2007). This range in prevalence could be as much a reflection of the different places where the studies were conducted as the different diagnostic approaches used in each study.

At first glance, the *Cryptosporidium spp*. found in feeder streams and raw water might have seemed to have had a wide range of possible sources. However, genotyping showed that most cryptosporidia in water samples were *C. ubiquitum* and novel genotypes (B, C, D and E), which were only present in water samples (Table 3.1.3). Only one water sample contained genotype A, which suggests that rodents are not a major source of *Cryptosporidium* contamination of water.

Rodents were mainly infected with the novel genotype A, and some field voles and "unknowns" were infected with previously reported genotypes UK E1, UK E7 and muskrat gt2. According to the PubMed GenBank database, UKE1 (GQ183522) and Muskrat gt 2 (GQ183516) have only been previously isolated from raw water (Chalmers et al., 2010), while UK E7 (HQ822143, HQ822135) has been previously found in surface river water and a Holstein calf. (Robinson et al., 2011).

On the other hand, the main species found in feeder streams and to a lesser extent in raw water was *C. ubiquitum,* which is considered to have the widest geographic distribution and host range of all *Cryptosporidium* species, having been previously described in wild and domestic ruminants, rodents, racoons and primates including humans. *C. ubiquitum* as a zoonotic infection has been previously described (Fayer et al., 2010). Molecular analysis, however, has recently shown a possible host adaptation; subtypes XIIa has been found almost exclusively in ruminants worldwide, while subtypes XIIb - XIIf have been isolated from rodents in USA and Europe. Humans have been found to harbour subtypes XIIa – XIId, which suggests ruminants and rodents may be the main sources of infection (Li et al., 2014). *C. ubiquitum* has been commonly isolated from recreational, catchment and drinking waters around the world and could also represent a potential risk to humans and other animals (Nichols et al., 2010, Van Dyke et al., 2012, Nolan et al., 2013, Galvan et al., 2014, Koehler et al., 2016).

C. ubiquitum was detected in only one wood mouse sample in this study, however, suggesting that this species is not abundant in wild rodents in this area and therefore the origin of contamination is most likely elsewhere. None of the livestock samples were positive for *C. ubiquitum*; however the sample size was too small to draw conclusions regarding the prevalence of *C. ubiquitum* in the sheep

and cattle. Thus the origin of potentially zoonotic *C. ubiquitum* in Llyn Cowlyd water remains unknown but clearly associated with feeder streams.

The most common species of *Cryptosporidium* found in raw water was yet another novel genotype (novel D): obviously as a novel genotype it has not been linked to human infection and its zoonotic potential is unknown, however, it could be argued that not having been associated with human illness makes it unlikely to be a human pathogen. This species was most likely responsible for the summer peak observed in 2015 and potentially for the peaks observed in previous years. That it was not found in any wildlife tested or in feeder streams suggests that its host might be aquatic: either fish or, perhaps less likely, aquatic birds. This obviously requires further research. If this is the case, the zoonotic potential of this genotype would be minimal as no species of *Cryptosporidium* from fish or birds have been linked to human disease as yet (Fayer, 2010, Ryan, 2010). It would be interesting to compare the novel sequences found in water (D) with known fish species and genotypes in order to see if they align more closely with cryptosporidia from fish than mammals.

C. parvum is the most common zoonotic cryptosporidial species infecting humans (the other main species found in humans, *C. hominis*, is not zoonotic – ie is found only in primates), and has been reported in a multitude of hosts, including livestock and wild rodents. Its prevalence in livestock has a wide range, from 2 to 70%, with higher ranges and susceptibility (16%-70%) for animals under 4 months old (Castro-Hermida et al., 2007, Mahfouz et al., 2014, Smith et al., 2014, Shafiq et al., 2015, Mirhashemi et al., 2016, Romero-Salas et al., 2016). *C. parvum* oocysts were found in two sheep samples and one raw water sample, making sheep a potential contributor of oocysts found in water. According to DCWW regulations, livestock should be kept out of the reservoir area, but as observed during this study, this was not always complied with and sheep were found regularly along the banks of the lake, and of course had free access to feeder streams. Infections are most commonly associated with young livestock; perhaps the low prevalence found in this study was due to the age of the animals at the time, as no lambs under 4 months old were seen.

A study by Wells et al. (2015a) found an unusually high prevalence in adult cattle and sheep in Scotland. However, the authors attributed this prevalence in part to an increased sensitivity in the method used to concentrate *Cryptosporidium* oocysts from adult faecal samples rather than a true higher prevalence within the sampled farms. This same method was used for this study but no other method was used for comparison.

Table 3.1.3 Summary of the origin of *Cryptosporidium* species and genotypes obtained in this study. This table shows the isolated species or genotype of *Cryptosporidium* regardless of samples with more than one species/genotype.

Species/sample	Total tested	*Cryptosporidium spp.* +	*C parvum*	*C ubiquitum*	*C ryanae*	*C bovis*	*Novel A*	*Novel D*	*muskrat*	*UK E1, UK E7*	*Other novel (B, C, E)*
FIELD VOLE	41	22					13		5	4	
UNKNOWN	20	3					2		1		
WOODMOUSE	35	10	*2*	*1*			*7*				
SHEEP CATTLE	10 1	3 1	*2*		*1*	*1*					
FEEDER STREAMS	72	19		11							11
RAW WATER	49	22	*1*	*5*			*1*	*16*			*3*

The reported prevalence of *C. parvum* in wild and peridomestic rodents varies widely, from 11% to 73% (Chalmers et al., 1997, Chilvers et al., 1998, Sinski et al., 1998, Sturdee et al., 1999, Torres et al., 2000, Bajer et al., 2003 Feng, 2010, Perec-Matysiak et al., 2015), presumably with host and location. In this study only two samples, both from woodmice, contained *C. parvum* which perhaps points towards a host adaptation of this species. *C. parvum* has not been previously reported in woodmice or field voles but has been in bank voles (Chalmers et al., 1997, Bull et al., 1998, Torres et al., 2000, Bajer et al., 2003) and common shrews (Sinski et al., 1993, Sturdee et al., 1999, Torres et al., 2000). Although both bank voles and shrews were sampled in this study, one of each was caught, and both tested negative for *Cryptosporidium*. The traps used had small escape holes for shrews (and through which very young rodents would also be able to escape), so it is likely that a proportion of the 'unknowns' in this study were shrews.

The zoonotic potential of the small amounts of *C. parvum* found in this study remains unknown. *C. parvum* has eleven recognised subtypes (IIa-I, IIk, IIl) based on sequence analysis of the GP60 glycoprotein gene, and different subtypes appear to have different host ranges. Subtypes, for example, IIb, IIc and IIe have only been found in humans (Xiao, 2010). In studies where *C. parvum* isolated from wildlife or livestock was thought to be responsible for human outbreaks, further characterisation showed that not all of the isolates belonged to the genotypes found in humans and the initially assumed importance of zoonotic transmission in these outbreaks became less clear (Perz and Le Blancq, 2001, Chalmers et al., 2002). Unfortunately, further characterisation of the *C. parvum* found in woodmice, sheep and water samples in this study was not possible as the PCRs for GP60 were unsuccessful and no DNA was left to repeat the assay.

In summary, this study has not conclusively identified the source of the cryptosporidial oocysts reported in water taken from Llyn Cowlyd. However, it has narrowed down the likely sources, and furthermore, the results suggest that the zoonotic potential of most of these oocysts is probably low. Further studies can be done with a focus on those samples most likely to yield useful data and sequences that can be used for phylogenetic analysis rather than simply genotyping. In the case of livestock samples, collection of faeces should not be limited to those found within the wildlife trapping grids, as this reduced the availability of faeces: rather samples should be collected from anywhere in the catchment. Developing a strategy for sampling birds and fish in the reservoir could be of scientific interest in order to help establish

– or perhaps more likely rule out – birds as the origin of the novel genotype (D) found in the water.

More investigation could also be applied to non-biological factors, for example rainfall and water temperatures within the lake. DCWW has found annual peaks of *Cryptosporidium* oocysts in water since 2009, with highest number of oocysts recorded in mid to late July in 2009, early to mid-July in 2010 and late July in 2011 (DCWW, 2011), and in 2015 (during this study), the highest number of oocysts was observed at the beginning of August. These peaks may be related to rainfall as heavy rainfall at the reservoir between 2009 and 2011 was recorded 1 to 2 weeks prior to the observed peak of *Cryptosporidium* oocyst in the water (DCWW, 2011), and during this study the heaviest rain was experienced during 20th July to 2nd August (weeks 30 and 31). Previous studies have also described a positive association between heavy rainfall and increased oocyst in surface water in Scotland (Wells et al., 2015a), Norway (Kelman et al., 2011), Luxemburg (Burnet et al., 2015) and Thailand (Chuah et al., 2016) as well as other sources of surface waters around the world (Young et al., 2015). The peak recorded by DCWW was seen at the same time as most cryptosporidia were detected in feeder streams and raw water sampled for this project: week 32 (August 3rd to August 9th).

In contrast the highest prevalence of *Cryptosporidium* in wild rodents was observed during week 36 (August 31st to September 6th) and in livestock at week 26 (June 22nd to June 28th) and week 36. These observations all suggest a more likely relationship between rainfall events and increase in oocysts observed in water than an increase in animal shedding and oocysts getting into the water. Rainfall, of course, not only increases wash off from the immediate catchment, but the churning of water through increased flow from feeder streams.

Another hypothesis, yet to be investigated, involves impact of water temperature changes – and in particular the gradual heating of the lake during the summer, and the subsequent disturbance in water layers due to the currents/columns formed. These columns might cause oocysts in deeper layers to be carried to the surface, and from there to enter the raw water intake.

Investigation of *Giardia* was not the initial aim of this study, but yielded some interesting results. *Giardia spp.* DNA was detected in samples from 27% field voles, 20% sheep and 51% feeder streams. No woodmice (*A. sylvaticus*) samples were positive, however. Previous studies have demonstrated *Giardia spp.* in bank voles (Bajer et al., 2003, Bajer, 2008b, Perec-Matysiak

et al., 2015), and uncharacterised *Giardia*-like cysts in woodmice (Karanis et al., 1996, Lohmus and Albihn, 2013). A further study by Haiba (1956) showed that experimental infection of woodmice with *G. duodenalis* cysts from human patients did not produce colonization in the rodents . Other species of wild rodents have been reported as having higher prevalences of *Giardia* excretion than found in this study, ranging from 13 to 100% (Chilvers et al., 1998, Ito and Itagaki, 2003, Bajer et al., 2003, Bajer, 2008b, Kilonzo et al., 2013, Fernandez-Alvarez et al., 2014). This, however, appears to be the first study of *Giardia* in field voles (*M. agrestis*). In addition to differences between host species and geography, in this study the PCR used targeted mainly *G duodenalis* (the only *Giardia* species known to infect humans) and would not be expected to have detected other species. This will be discussed further in the next chapter.

The livestock sample size was too small to draw any firm conclusions regarding prevalence of *Giardia*, however, the prevalence found in sheep was similar to that reported in other studies where 20-30%, with some showing a wider 1.3% to 89% prevalence, were found (Gomez-Munoz et al., 2012, Yang et al., 2014, Yang et al., 2015, Wang et al., 2016). No *Giardia* was detected in the one cattle sample examined in this study, and previous studies have found infection of cattle to be less abundant than in sheep, with reported prevalence of 1% to 33% (Minetti et al., 2014, Stojecki et al., 2015, Li et al., 2016). In both livestock faeces, as for cryptosporidiosis, infection rates are higher in younger animals (<2 months) than in adults (Castro-Hermida et al., 2007, Bajer, 2008b, Paz e Silva et al., 2014, Liu et al., 2015, Ye et al., 2015), and as noted above, there were few young animals present during this study. Future studies of giardiasis might be conducted earlier in the year to determine any seasonal differences in contamination loads.

Previous studies have shown that *Giardia* oocysts are commonly found in surface waters and abundance is thought to be linked to rainfall events when faecal matter can reach the water through run off (Burnet et al., 2015, Daniels et al., 2015, Mahmoudi et al., 2015). In this study, the peak in *Giardia* detection in wild rodents was at weeks 31-32 (July 27th to August 9th), while in feeder stream samples it was during weeks 36 – 37 (August 31st to September 13th). The only livestock positive samples for *Giardia* were taken at weeks 26 – 27 (June 22nd to July 5th).

Further investigation of the ecology and zoonotic potential of *Giardia* in the study area would require better molecular characterisation of the *Giardia* samples. Such investigation and a discussion of its implications are described in section 3.2.

Limitations of the study

There were three main limitations with this study; the first one was the unexpectedly small population of rodents found in the study area, even though this was not designed to be an ecological survey of the rodent population, the trapping outcome showed the limited number of animals inhabiting the area surrounding Llyn Cowlyd. Future studies of wildlife in the area could help determine if the low population size was normal or if there were other factors influencing populations during the time of the study.

One reason for smaller than expected populations might be the weather, which was the other main limitation of the study. As well as possibly reducing rodent activity, the wet weather interfered with the trapping scheme as some traps were triggered or moved by runoff from the hills.

The presence of larger animals such as sheep, cattle and humans also affected the study, particularly when traps were tipped over, triggered, moved or tampered with. On the other hand, it might have been useful to have collected more farm animal samples – and if this project were to be repeated, more livestock sampling should be undertaken.

This study involved a network of stakeholders, mainly from DCWW and the PHW *Cryptosporidium* reference laboratories, as well as the UoL. The nature and investment of the participants, myself included, as well as the area where the project took place, meant that collection and processing of samples was done without interference from third parties, which allowed a more focused study although somewhat limited to this particular area. If inferences are to be made about other reservoirs in the area, however, a much bigger study, involving more stakeholders would have to be conducted.

The results from this study show that even though *Cryptosporidium* was found in wildlife, the genotypes found were mostly non-zoonotic and therefore pose little threat to human health; on the other hand, the presence of sheep and cattle within the reservoir may increase the risk of contamination of the water with potentially zoonotic oocysts, particularly after heavy rainfall events.

The zoonotic species *C. parvum* was found during this study in raw water, sheep and a woodmouse, however, further characterisation is needed determine the true zoonotic

potential of the *C. parvum* oocysts detected in Llyn Cowlyd. Interestingly, the most common type of *Cryptosporidium* found in raw water was a novel genotype not (yet) linked to human infection. Future studies should look at fish and birds as the potential source of this genotype.

Similarly, not all *Giardia* genotypes are zoonotic, and the relative input of livestock and wildlife into *Giardia* prevalence in water found in this study is not clear. However, the presence of cysts in sheep faeces found along the reservoir could indicate a potential risk for other animals including humans. Further studies might include the lambing season (February-March) to determine whether the presence of younger animals within the reservoir affects the infection rates of protozoa in wildlife and later in the year in water. Most important, however, is to attempt to characterise further the *Giardia* detected, and this is described in section 3.2.

3.2. Genetic characterisation of Giardia species found in wild rodents, livestock and feeder streams in Llyn Cowlyd

3.2.1. Introduction

Giardia is a flagellated protozoan first described by Van Leeuwenhoek in 1681. It is responsible for approximately 280 million cases of diarrhoea worldwide every year (Lane and Lloyd, 2002), although symptoms may also include abdominal cramps, nausea, malaise and malabsorption (Ortega and Adam, 1997). It has also been linked to post-infection irritable bowel syndrome (IBS) (Hanevik et al., 2009).

As discussed in sections 1.2.3 and 3.1, there are currently six species of *Giardia,* initially based on light microscopy examination and more recently confirmed by 16S rRNA sequencing: *G. duodenalis, G. agilis* (amphibians)*, G. psittaci, G. ardeae* (both in birds), *G. muris* and *G. microti* (both in rodents). *G. duodenalis* has a wide host range and is the only species found in both human and other animals (Adam, 2001). However, the lack of molecular characterisation of *Giardia* found in both human and non-human animals has led to an overestimation of the zoonotic potential of this protozoan (Pacha et al., 1987, Monis and Thompson, 2003).

The methods used for identification of *Giardia* species and assemblages differ depending on the gene used, the region of the gene examined and the method of analysis (Wielinga and Thompson, 2007). There are 16 target genes frequently used for identification of *Giardia* species and within species variations (assemblages). Glutamate dehydrogenase (*gdh*), triose phosphate isomerase (*tpi*), β–giardin (*bg*), elongation factor 1-α (*ef-1*), 18S rRNA and ITS regions 5.8S rDNA can be used to genotype all assemblages of *G. duodenalis* as well as identify *G. muris;* in addition, markers targeting *tpi, gdh,* 18S rRNA and 5.8 rDNA genes can also identify *G. microti* and/or *G. ardeae* and *G. agilis*. Other genes such as *Mlh 1*, *ferrodixin*, *histone H2B*, *histone H4*, *actin, α–tubulin*, *chaperonin 60* and Intergenic ribosomal spacers are mainly used to genotype assemblages A and B of *G. duodenalis* (Plutzer et al., 2010, Caccio and Sprong, 2011).

Phylogenetic comparison of *dgh, tpi* and *bg* loci suggests seven assemblages for *G. duodenalis*, which largely correspond with most common hosts and old species names: A (*G. duodenalis*), B (*G. enterica*), C, D (*G. canis*), E (*G. bovis*), F (*G. cati*) and G (*G. simondi*) (Monis et al., 2009). An

eighth assemblage (H) has been suggested associated with marine vertebrates (Lasek-Nesselquist et al., 2010).

It is generally accepted that only assemblages A and B are linked to human diarrhoea. Assemblages C, D, F and E have been found sporadically in human cases but it is unclear whether they were the cause of disease or accidental findings (Sprong et al., 2009).

Aside from humans, assemblage A has been isolated from cats, dogs, domestic livestock and several species of wildlife. In rare cases, assemblage B has also been found in wild animals and dogs, although it has been commonly isolated from captive non-human primates, which are thought to have been infected through exposure to humans (Sprong et al., 2009, Plutzer et al., 2010). Some studies also describe finding *G. duodenalis* cysts of assemblages A and B in flies and wild bird droppings, which could be a contributing factor to parasite dissemination (Szostakowska et al., 2004, Plutzer and Tomor, 2009).

Further analysis of assemblage A has led to the description of three genotypes: AI, found mostly in livestock and pets, as well as humans, AII, found mostly in humans and AIII, found almost exclusively in wild hoofed animals (Caccio et al., 2008). The great genetic diversity within assemblage B has made identification of subgroups very difficult. Allozyme electrophoresis of human and other animal samples showed four distinct clusters within assemblage B: BIII and BIV were identified in humans while BI and BII were only obtained from monkeys and a dog (Monis et al., 2003). Due to the genetic heterogeneity of this assembly, further polymorphic genes have been suggested as being potentially of use in genotyping assays (Wielinga et al., 2015).

Some studies have suggested an association between different assemblages and clinical signs in humans; however, the evidence for this is conflicting. Sahagun *et al.* (2008) and Haque *et al. (2005)* found a correlation between assemblage AII and symptomatic infections, while assemblage B was linked to asymptomatic infections, Homan and Mank (2001) found that assemblage B was linked to persistent diarrhoea while assemblage A was linked to intermittent presentation. More recently, Mahdy *et al.* (2009) and Kohli *et al.* (2008) found no difference in clinical presentation or that assemblage B was linked to symptomatic disease when both assemblages were present within the population.

The role of wildlife, and in particular wild rodents, in the epidemiology of giardiasis in humans and livestock is unclear: some studies have identified *G. duodenalis, G. muris* and *G. microti* using morphometric or sequencing methods in wild and peridomestic rodents (van Keulen et al., 1998, van Keulen et al., 2002, Ito and Itagaki, 2003, Perec-Matysiak et al., 2015) and only two studies have reported potentially zoonotic assemblages A or B in naturally infected rodent populations (Bajer, 2008a, Fernandez-Alvarez et al., 2014). Experimental infection of wild and laboratory rodents with *G. duodenalis* obtained from human patients showed the potential of rodents to act as source of human giardiasis (Haiba, 1956, Cheng et al., 1993), however there have not been any reports linking human outbreaks and rodents.

The aim of this study was to compare the sequences of *Giardia duodenalis* from wild rodents, livestock and feeder streams sampled in Llyn Cowlyd reservoir between June and September 2014, with sequences available in GenBank to determine the assemblage and clade they belonged to and thereby infer their potential zoonotic risk.

3.2.2. Methods

Animal and water samples

G. duodenalis amplicons from animal (field voles and sheep) and water samples collected in Llyn Cowlyd between June and September 2014, were obtained by amplification of a 204 bp region of the *bg* gene using real-time PCR (Rotor-Gene®, Qiagen) amplification following the conditions specified by Chalmers et al. (Chalmers, 2015). The primers used were BGF2 (GAGGCCCTCAAGAGCCTGAA), BGF2R2 (ACACTCGACGAGCTTCGTGTT) and BG2T (VIC-ATCGAGAAGGAGACGATCGC-MGB-NFQ). Four PCR replicates were carried out for each water sample. Amplicons were purified using a QIAquick PCR purification kit (QAGEN) following the manufacturer's instructions and sequenced by Source Bioscience. Amplification and purification was done by Dr. Kristin Elwin at the CRU (Swansea).

Sequence analysis was conducted using Chromas Pro1.4 (Technelysium Pty. Ltd) by editing and assembling nucleotide sequences and compared against all sequences on Genbank using the Basic Local Alignment Sequence Tool (BLAST) to determine their assemblage and location within a phylogenetic tree constructed using both data.

NCBI database sequences

Giardia duodenalis sequences based on *bg* locus in the NCBI nucleotide database were collected and curated in a local database. All non-redundant sequences were kept for analysis while those that contained the identical nucleotide sequences were eliminated.

Sequence analysis

Sequence analysis was performed by Dr. Richard Eemes at the University of Nottingham using PhyMl 3.0 (CNRS - Universite Montpellier II) which is based on a maximum-likelihood algorithm to infer phylogenetic tree topology. Sequences collected from animal and water samples, as well as those gathered from the NCBI database were compared and trees constructed. Bootstrap values were calculated by through a 1000 replicates and final phylogenetic trees were edited using FigTree v 1.4.2 software.

3.2.3. Results

A total of 173 faecal and water samples were screened for *G. duodenalis* using the *bg* gene. Overall, *Giardia* DNA was detected through RT-PCR in 29% (50) of the samples (Fig. 3.2.1).

A total of 684 NCBI nucleotide sequences were reported as *G. duodenalis* on GenBank, of which only 251 were non-redundant.

Comparison of amplicons with published sequences of *G. duodenalis* enabled the majority of amplicons to be placed into a small number of distinct clades (Fig 3.2.2, and summarised in Table 3.2.1). The most common assemblage detected was E (72%, including two apparent mixed samples), which was found in feeder stream water and sheep. The potentially zoonotic assemblage A was found in three water samples (one mixed with E), but not in any potential animal sources. All positive field vole (*M. agrestis*) samples contained a novel assemblage, not previously reported using the *bg* gene, when compared to the sequences in the GenBank database. Two water samples also contained a sequence similar to this novel genotype, in one case mixed with and E sequence. No other assemblages were found in any of the samples. (Table 3.2.1, Figure 3.2.2).

Table 3.2.1 *G. duodenalis* assemblages of field vole, sheep and feeder stream samples from Llyn Cowlyd.

Month	Species	Total sampled	*Giardia duodenalis* PCR +ve (%)	*G. duodenalis* assemblage
June	FIELD VOLE	4	0	
	SHEEP	3	2(66)	E (2)
	FEEDER STREAMS	18	9 (50)	E (9)
July	FIELD VOLE	15	3 (20)	Novel (3)
	FEEDER STREAMS	23	7 (30)	E (7)
August	FIELD VOLE	8	4 (50)	Novel (4)
	FEEDER STREAMS	17	9 (53)	E (7), Novel (1), A (1)
September	FIELD VOLE	14	4 (29)	Novel (4)
	FEEDER STREAMS	14	12(86)	E (9), A (1), E+A (1), E+Novel (1)

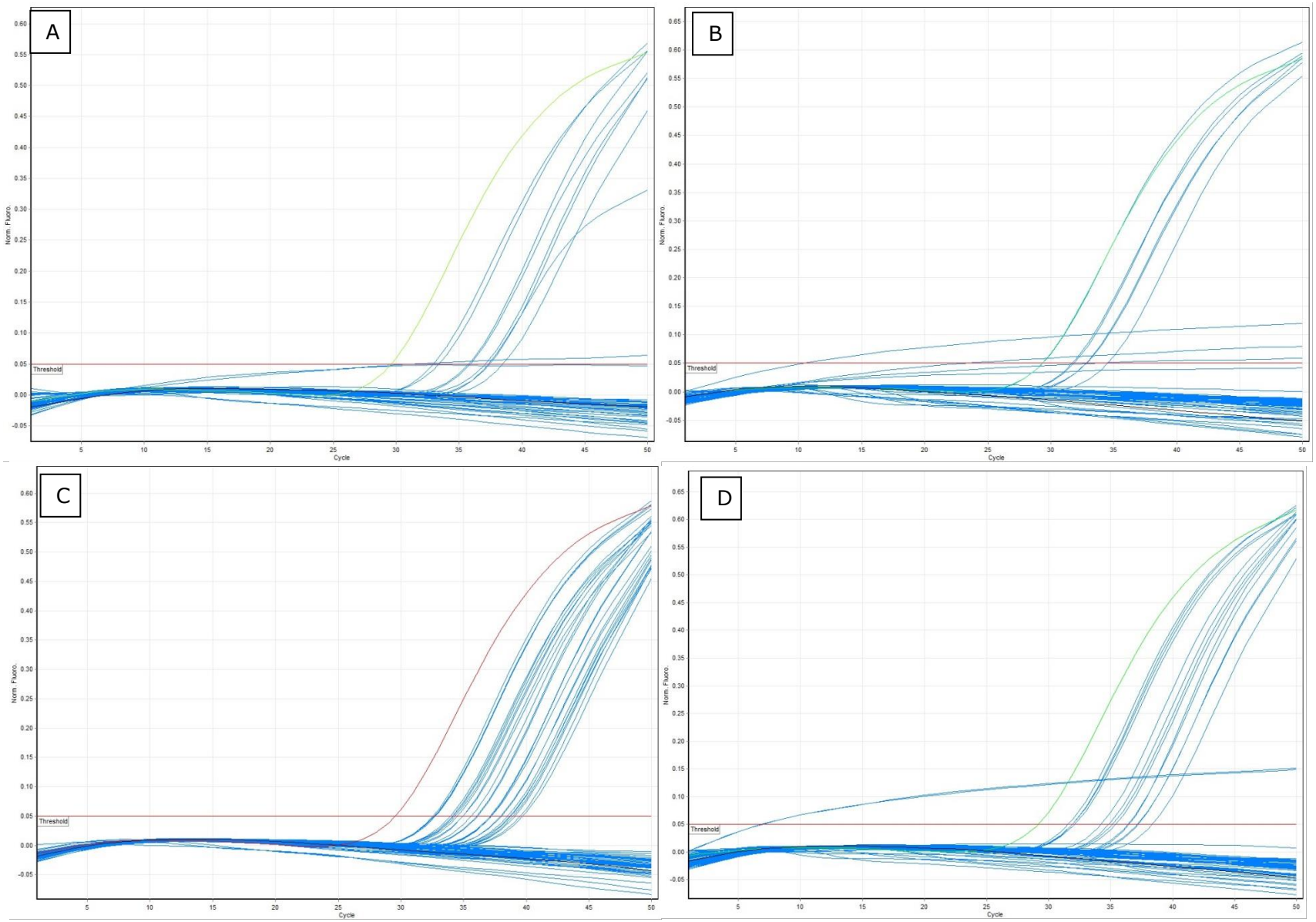

Figure 3.2.1 Detection of *G. duodenalis* DNA using RT-PCR in animal faecal (A and B) and feeder streams (C and D) samples. Samples clearly above the threshold of normalised fluorescence were considered positives. Positive controls are shown in a different colour than the rest of the samples.

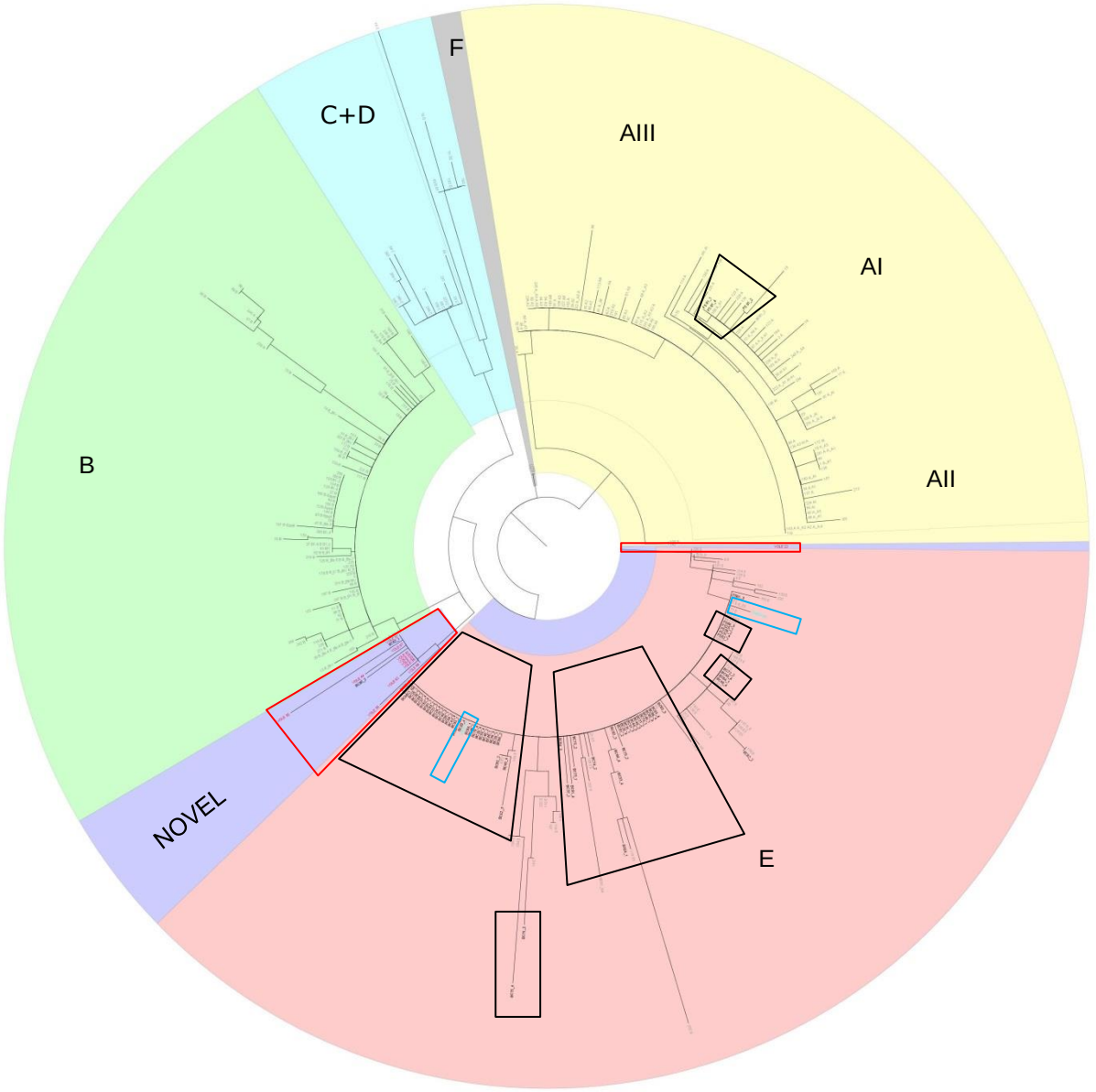

Figure 3.2.2 Phylogenetic relationship amongst assemblages A-F of *G. duodenalis* at the bg locus. Bootstrap values were calculated by the analysis of 1000 replicates. Position of field vole (red), sheep (blue) and feeder streams (black) samples are shown.

3.2.4. Discussion

Giardia duodenalis is responsible for at least 4000 reported cases of diarrhoeal disease every year in the UK, and at least 16,000 in the EU (ECDC, 2014). These protozoa can also cause chronic fatigue, malabsorption and dehydration in humans. In non-human animals, cysts have been found in the faeces of ruminants, pigs, dogs, cats and small mammals. Infection is mostly without clinical signs in adults but can cause watery diarrhoea, decreased weight gain and malabsorption in younger animals (Geurden and Olson, 2011).

The *bg* gene of *G. duodenalis* has been considered a useful locus to amplify as giardins are proteins found in the ventral disk of the trophozoites and are considered to be unique to *Giardia* (Holberton et al., 1988, Faubert, 2000, Adam, 2001). The main aim of this study was to identify potentially zoonotic species of *Giardia* in water as well as in wildlife and livestock surrounding the Llyn Cowlyd reservoir, in order to infer the true origin of any pathogenic cysts found in water.

Assemblages A and B have been extensively reported in surface waters around the world, and have been considered a potential source of infection for humans (Adamska, 2015, Burnet et al., 2015, Mahmoudi et al., 2015). Some studies have implicated non-human animals as the contamination source of water and therefore to humans. However, infection of wildlife and livestock from human contamination has not been explored fully. Water exposed to agricultural waste discharge or improperly treated human sewage can carry pathogenic cysts into the fresh water and marine environments where they can be ingested by marine mammals and shellfish. These spill-over species can then act as a source of human infection (Appelbee et al., 2005).

In this study, water samples contained mainly assemblage E and only 3 samples contained assemblage A (one mixed with assemblage E). Sequences from positive sheep samples were also both assemblage E. Although only a small number of livestock samples were examined, it seems likely that livestock, in this case sheep, were the source of the majority of cysts found in water. Assemblage E is considered exclusive to artiodactyls (Ey et al., 1997), and therefore of limited zoonotic potential. Finding cysts belonging to this assemblage is common in areas grazed by sheep and other hoofed animals and represents little to no risk to humans. It would seem that it is also of low infectivity to wild rodents since none of the rodent samples contained this assemblage either.

Interestingly, the *Giardia* sequences obtained from rodent samples were only from field voles, and formed two apparent clades clearly separate from any of the assemblages previously described using the *bg* gene in the GenBank database. Two water samples also contained similar sequences to those obtained from the rodent samples. This suggests that run-off can carry faecal matter from rodents into the feeder streams, and presumably also to the reservoir. However, the risk to humans from this genotype is likely to be small as it has never been described before in human cases and, at least in this system, dilution in the reservoir is likely to be great.

More work is required, ideally using other loci, in order to more fully characterise the apparently novel *Giardia* assemblage detected in field voles. The PCR used in this study would not detect *G. muris*, but is likely to detect assemblage G (previously known as *G microti*). When constructing the phylogenetic relationship tree, once redundant sequences were eliminated, assemblage G appeared to be within the E clade. Comparison of the novel rodent-derived sequences in this study with published assemblage G sequences gave relatively low similarities (<96% BLAST identity). Thus, although further loci should be studied, all the evidence so far points to these rodents *Giardia* as belonging to novel assemblages.

Traub *et al*. (2004) emphasised the importance of using more than one locus to genotype *G. duodenalis*, describing how human isolates typed using only the SSU-rDNA appeared to be infected with genotype C and D but when typed using *tpi* and *ef1-a* appeared more likely to be infected with assemblages A and B. Because in this study rodent samples were not tested using other loci, future work should involve determining the novelty of these assemblages targeting other genes and comparing them to known sequences of *G. duodenalis* previously found in rodents and other animals.

So, where did the assemblage A found in three water samples come from? Sheep and cattle can harbour assemblage A, although in this study we only found assemblage E. It has been suggested that younger animals are more susceptible to assemblage A while older, more immunologically mature animals are more resistant to this assemblage and are more commonly infected with the host-adapted assemblage E (Uehlinger et al., 2011). In order to establish if this is the case within the Llyn Cowlyd reservoir, further studies would have to be conducted encompassing periods when lambs were present, especially considering *Giardia* cysts can remain infective in water and soil for up to 11 weeks (Olson et al., 1999)

Other potential sources of assemblage A are humans and other domestic animals such as dogs. Based on the results of this study, these cannot be excluded as potential sources of zoonotic *Giardia* in feeder streams, as even though Llyn Cowlyd is not used for recreational purposes, multiple hikers and campers, some accompanied by one or more dogs, were observed in the catchment area during this study. It is also possible that contamination might have occurred a long way up stream in a lake from which one of the feeder streams came.

Limitations of the study

The same samples used for section 3.1 were used for this study, therefore the same limitations with the trapping and sampling scheme that were discussed in the previous section apply to this study.

Perhaps the biggest limitation specifically for this project was the use of only one locus to do the characterisation of the *Giardia* sequences. The decision to use only one locus was based on budget and prioritization of resources, as *Cryptosporidium* was the main priority for the majority of stakeholders involved. Nevertheless, evidence of non-zoonotic, novel assemblages found in wild rodents in the study area showed the importance of genotyping studies that can help allocate future attempts to control water contamination.

In ecological terms, a further limitation came from the decision to only test for the zoonotic *G duodenalis*. Other species of *Giardia* have been reported in wild rodents, and it would be interesting to understand the relationship between these and *G. duodenalis* in terms of multiple infection or exclusion.

In conclusion, this study showed that wild rodents are not likely to be the source of potentially zoonotic giardia found in water in Llyn Cowlyd reservoir, but humans and livestock, particularly sheep could be likely sources. Additionally, field voles (but not other rodents) in this area excreted at least one apparently novel and host-adapted assemblage of *G. duodenalis* not previously described using the *bg*-gene locus.

4. *Campylobacter* species isolated from wild birds near dairy and poultry farms in Cheshire

4.1. Introduction

Campylobacter in humans has been associated with diarrhoeal disease and other gastroenteric disorders. Worldwide, approximately 96 million cases of gastrointestinal disease are reported each year with over 21,000 related deaths (WHO, 2015). In the EU alone, over nine million cases of *Campylobacter*-related diarrhoea are reported each year with an average incidence rate of 47 reported cases /100,000 inhabitants, meaning one in fifty people will be diagnosed with campylobacteriosis each year (Pires et al., 2010, Havelaar et al., 2013). In the UK over 66,000 cases of campylobacteriosis are reported each year; however it has been estimated that the actual number of cases could be up to 500,000 due to under reporting (Tam et al., 2012).

The most common source of human campylobacteriosis is chicken, either through consumption of undercooked poultry, handling or preparing broiler meat, or cross contamination from raw meat (Humphrey et al., 2007, Wilson et al., 2008, Horrocks et al., 2009, Mullner et al., 2009,DEFRA, 2010, EFSA, 2010). A recent survey conducted in the UK also found *C. jejuni* on the outer packages of supermarket poultry products, which implies an extra source of *Campylobacter* for unsuspecting consumers (Jorgensen et al., 2015).

Red meat consumption from cattle, sheep and pork has also been implicated in human campylobacteriosis outbreaks in New Zealand (Rob Lake et al., 2007), the UK (Frost et al., 2002, Wilson et al., 2008, Sheppard et al., 2009), the United States (Friedman et al., 2004) and the Netherlands (Mughini Gras et al., 2012). As with poultry products, the outer packaging of raw meat products has also been found to be contaminated with *C. jejuni* and *C. coli* (Burgess et al., 2005).

Other food sources implicated in human outbreaks include dairy products such as milk (Doyle and Roman, 1982, Lovett et al., 1983, Korlath et al., 1985, Heuvelink et al., 2009, Davis et al., 2016) cheese and ice cream (Taylor et al., 2013), pasteurized milk (Lighton et al., 1991, Riordan et al., 1993, Palmer and McGuirk, 1995), fresh fruits and vegetables, particularly those “ready-to-eat” (Neimann et al., 2003, Little and Gillespie, 2008, Verhoeff-Bakkenes et al., 2011), contaminated water sources

(Kuusi, 2004, O'Reilly et al., 2007) and bottled water (Evans et al., 2003, Mughini-Gras et al., 2014, MacDonald et al., 2015).

Environmental sources of infection also have a role in the epidemiology of campylobacteriosis. Direct contact with contaminated playground surfaces (e.g. soil, grass, plastic, wood, concrete, etc.), especially contaminated with wild birds faeces, have been linked to cases in children sharing or playing in the same areas (French et al., 2009, Ramonaite et al., 2014). One study in the UK suggested that the proportion of cases attributable to wild bird faecal contamination could be almost 100,000 over a ten year period, making wild birds potentially a considerable source of *Campylobacter* infections (Cody et al., 2015). Pets (Wolfs et al., 2001, Tenkate and Stafford, 2002, Mughini Gras et al., 2013, MacDonald et al., 2015) and farm animals, through leisure activities or occupational exposure can be sources of *Campylobacter* as well (Taylor et al., 2001, Pintar et al., 2015). In children under 5 years old living in rural areas, direct contact with animals and a contaminated environment is more important as a source of infection than food (particularly retail chicken and meat), which is a more important source of infection in urban children (Strachan et al., 2009).

There are further possible sources of infection that have not yet been directly proven to be sources of human and/or livestock infection. For example, flies have been shown to be capable of carrying *Campylobacter* for 24 hours after exposure (Nichols, 2005, Evers et al., 2016), although they are not able to amplify the microorganism, making them only mechanical vectors (Gill et al., 2016). There is also a potential for *C. jejuni* to be transmitted sexually between humans, although further studies are needed to confirm this (Gaudreau et al., 2015).

So how does *Campylobacter* enter the food chain? Livestock can become colonised prior to slaughter or contaminated during or after slaughter. *Campylobacter*-free poultry flocks have been shown to become colonized within a week of the microorganism being introduced into the flock (Evans and Sayers, 2000), which shows how rapidly horizontal transmission can occur. The natural source of the *Campylobacter*, however, seems to be unclear: vertical transmission is highly unlikely (Callicott et al., 2006, Battersby et al., 2016) and the evidence to the contrary is not conclusive (Cox et al., 2012, Marin et al., 2015). Therefore, the source is most likely to be environmental, with water, wildlife, workers equipment and fomites as the most likely candidates (Annan-Prah and Janc, 1988, Pearson et al., 1993, Gregory et al., 1997, Lee and Newell, 2006, Humphrey et al., 2007).

For other livestock, wild birds have been identified as vehicles and a source of *C. jejuni* (Sippy et al., 2012) and in some cases, when access of birds to animal feed was restricted a decrease in *C. jejuni* in the herd was observed (Wesley et al., 2000).

During transportation to slaughter, broiler flocks can become contaminated due to inadequately cleaned vehicles and crates (Stern et al., 1995, Hansson et al., 2005) and the stress related to transport, which has been shown to increase shedding of faecal matter and therefore increase the possibility of contamination and transmission (Mulder, 1999, Whyte et al., 2001, Herman et al., 2003).

In most chicken processing plants, birds are shackled, killed, scalded, defeathered, eviscerated, washed, cooled and packaged. Scalding and defeathering have been shown to be potential areas where cross-contamination may occur as well as "carry-over" effect to *Campylobacter*-free animals from equipment used after processing colonised animals (Newell et al., 2001, Keener et al., 2004, Mullner et al., 2009, Mughini Gras et al., 2012) Contamination of carcasses with gastrointestinal contents during evisceration processes is also possible (Horrocks et al., 2009, Seliwiorstow et al., 2016). After slaughter, cross contamination can occur inside the carcass chillers, particularly if water chillers are used instead of air chillers (Sanchez et al., 2002).

Because poultry plants process thousands of chickens every day, with some processing up to 26,000 birds per hour, cleaning and disinfecting between flocks might not be possible or can be insufficient to eliminate bacteria; organic acids, crust freezing, ice blasting steam, ionising radiation and ultrasound are some of the methods currently used to help eliminate any remaining bacteria on the carcasses (Keener et al., 2004, Gregory-Kumar, 2015).

After processing, *Campylobacter* can survive on carcasses that have been stored in both air or carbon dioxide atmospheres (CO_2 is used to extend the shelf life of poultry products), under low temperatures which could potentially lead to cross contamination, and in commercial and domestic kitchens where utensils and chopping boards can act as vehicles of *Campylobacter* and other pathogens (Gorman et al., 2002, Guyard-Nicodeme et al., 2013, Lopez et al., 2015).

Dairy products, particularly milk, can become contaminated during milking procedures due to the presence of *Campylobacter* in the intestinal tract of infected cows, even when udders are washed and dried beforehand (Robinson and Jones, 1981, Humphrey and Beckett, 1987). Although rare, *C.*

jejuni excreted directly from asymptomatic or mastitis–free udders can be another source of contamination for raw milk (Hutchinson et al., 1985, Orr et al., 1995, Bianchini et al., 2014a). In the case of pasteurised milk, contamination occurs generally after leaving the processing plants and in some cases has been linked to birds pecking the milk bottles (Lighton et al., 1991, Riordan et al., 1993, Palmer and McGuirk, 1995, Taylor et al., 2013).

The use of molecular characterisation to determine the source of *Campylobacter* infections is important as it can influence control and prevention strategies. Subtyping for *Campylobacter* has evolved from methods based on phenotypic properties such as serotyping and electrophoretic mobility of enzymes (MLEE) to DNA-based methods, of which the most extensively used is multi-locus sequence typing (MLST). MLST is a PCR-based method which assigns an allele number to the sequenced PCR products for each of 7 loci representing house-keeping genes (*asp, glnA, gltA, glyA, pgm, uncA,* tkt) based on a complete match to an allele in the global PubMLST database. The combination of these seven allele numbers is then assigned a sequence type (ST) and if four or more alleles are shared by different STs these then are set into a Clonal Complex (CC) (Taboada et al., 2013).

MLST STs and CCs determination has linked some genotypes with certain hosts and environments, showing that not all isolates belonging to the same species of *Campylobacter* are linked to human disease. This means that the role of wildlife in human and livestock infection while still unclear, can be investigated. Questions that need to be answered, if control protocols are to be prioritised, include: what are the roles of various wildlife species in the transmission of *Campylobacter* to humans and livestock? What is the relationship between isolates obtained from wild animals and those obtained from environment? When the same types are found in two hosts, what is the main direction of transmission?

Of all wild animals capable of carrying *Campylobacter*, wild birds are still regarded as most likely candidates to be the source of infection in the farm environment: their ubiquity in rural and urban areas as well as their ability to fly over large ranges make them a perfect vehicle for dispersal of pathogens, which is why they were chosen as the focus for this study. Furthermore, as chickens are birds, it might be expected that any host barriers might be less between wild birds and poultry. On the other hand, few studies have been undertaken to test host range. One experimental study showed probable host adaptation amongst *C. jejuni* isolates: European robins (*Erithacus* rubecula) inoculated with *C. jejuni* isolates from human patients and from other wild birds were colonized only

by the bird isolates (and developed decreased body mass), although the human isolates of *C. jejuni* could be recovered from robin faeces for a short period after inoculation (Waldenstrom et al., 2010). The study described in this chapter was part of a lager programme of work investigating the transmission of campylobacter on farms, in the environment and, in particular, to people. Its specific aims were:

1. To investigate the prevalence and sequence types of *Campylobacter* spp. in wild birds near a dairy and a poultry farm in Cheshire
2. To compare any isolates to those previously found in livestock and environment on these two farms during the EMIDA and ESEI studies, and
3. To determine the presence of antimicrobial resistance in these wild bird campylobacter isolates.

4.2 Methods

Sample collection

Birds

A cross–sectional study of wild birds on two farms in Cheshire (Figure 4.1) was carried out between March 2015 and January 2016, with trapping conducted during three periods; spring (March-May), mid-summer (July) and winter (November-January). These periods were chosen to get as wide a range of birds as possible, taking account of migration patterns and welfare constraints, eg not sampling while birds are nesting or in wet/cold weather. Specific areas on each farm were selected based on observations of bird activity, again in order to obtain a range of species representative of the farms overall. Criteria included proximity to footpaths, observed bird activity and the ability to conceal mist nets against shrubs or other vegetation (Figure 4.2 A and B). Trapping was undertaken in collaboration with British Trust for Ornithology licensed bird ringers using mist nets. Ethics approval was granted by the appropriate panels and committees from the University of Liverpool and the University of Nottingham (VREC110, VREC112, SVMV 1747 160425, SVMV 1465150519).

A trap and release scheme was used. All birds were registered and ringed on site according to the BTO guidelines (Redfern and Clark, 2001) and put inside paper bags for no more than 30 minutes, during which they would normally defecate inside the bags. The faecal samples were then transported to the laboratory and collected from the bags using sterile swabs. Feral pigeons were provided by farm employees after pest control shooting was carried out, and samples were taken post-mortem from the distal intestine.

Environmental samples

Environmental samples from the same areas where the nets were set up were collected using the boot sock method, in which an absorbent shoe cover was placed over the boots while the birds were sampled or later that same day. This was done at least once for each farm. The shoe covers were then placed in re sealable bags to avoid cross contamination with other equipment and transported to the laboratory to be processed.

Bacterial culture

Faecal samples obtained from the paper bags, and those taken post mortem, were processed the same day by placing them in 2ml of *Campylobacter* enrichment supplement broth (Exeter), consisting of nutrient broth supplemented with sodium pyruvate (250mg/L), sodium metabisulphite (250mg/L), ferrous sulphate (205mg/L), trimethoprim (10mg/L), rifampicin (5mg/L), Polymyxin B (2500 iu/L), cefoperazone (15mg/L), amphotericin B (2mg/L) and 5% defibrinated horse blood (Humphrey, 1995), and incubated at 41°C under microaerobic conditions (N 80%, O_2 5%, CO_2 12%) in a variable atmosphere incubator (Don Whitley Scientific Ltd Shipley, UK) for 48 hours. Boot socks were soaked and agitated in 100ml of Exeter broth and left to rest for ten minutes, after which 3mL of the palpated broth were incubated in microaerobic conditions at 41°C for 48 hours.

A loopful of Exeter broth from both faecal and boot sock processed samples, was streaked out onto modified charcoal cefoperazone deoxycholate agar (mCCDA) supplemented with cefoperazone (32mg/L) and amphotericin (10mg/L) and incubated for 48 hours at 41°C as stated above. Three to four colonies with morphological characteristics of *Campylobacter* species (small 1-2 mm in diameter, round, greyish, flat or convex with irregular edges) were subcultured on two Columbia blood agar plates supplemented with 5% defibrinated horse blood, one of which was incubated in aerobic conditions at 30°C and the other at 41°C in microaerobic conditions both for 48 hours. Those microorganisms that grew in both microaerobic and aerobic conditions were considered to be *Arcobacter spp.* and were discarded. A loopful of bacterial growth from the plates that only grew under microaerobic conditions was added to a Microbank™ cryovial (Pro-Lab Ltd, Wirral UK) containing beads and cryopreservative and stored at -80°C for later use. Cryopreservation has been shown to be effective for long term storage of *Campylobacter* (Mills and Gherna, 1988, Gorman and Adley, 2004).

All culture media and antibiotic supplements used were from LabM Ltd (LabM Limited, Bury UK), and defibrinated horse blood was obtained from Thermo Scientific (Thermo Fisher Scientific, Leicestershire, UK).

Figure 4.1. Location A is the dairy farm. Location B is the poultry farm. Both were located in Cheshire, UK.

Figure 4.2. Location of the mist nets in the dairy farm (A) and the poultry farm (B)

Antimicrobial resistance

Isolates were tested for antimicrobial susceptibility by plating one bead from each Microbank tube onto a Columbia blood agar plate (Lab M) supplemented with 5% defibrinated horse blood (Thermo Scientific), and incubated for 48 hours at 41°C in microaerobic conditions. Antimicrobial resistance tests were conducted using the EUCAST standardised disk diffusion method (Matuschek et al., 2014). An inoculum with a density of a 0.5 McFarland standard of each isolate (prepared by resuspending in 3ml of saline solution approximately half a 5µl loopfool of bacterial culture) was plated using an automatic plate rotator onto Mueller-Hinton agar supplemented with 20mg/L of β–Nicotinamide adenine dinucleotide sodium salt (β-NAD) (Sigma-Aldrich) and 5% defibrinated horse blood.

Antimicrobial discs impregnated with ciprofloxacin (5 µg), erythromycin (15 µg) and tetracycline (30 µg) were applied on the agar surface no more than 15 minutes after inoculation of the plates.

The plates were incubated for 24 hours at 41°C in microaerobic conditions, after which inhibition zones were read. Plates with insufficient growth after 24 hours were incubated for a total of 48 hours after which they were read. To read the plates the zone edges were taken as the point showing no growth from the front of the plate with the lid removed.

In the case of *C. jejuni and C. coli,* susceptibility to the macrolides azithromycin and clarithromycin can be determined by using erythromycin, and tetracycline can be used to determine susceptibility to doxycycline (EUCAST, 2016).

Molecular characterisation

DNA was extracted by Chelex (Bio-Rad) method (Walsh et al., 1991) and isolates were confirmed to be *C. jejuni or C. coli* using a multiplex PCR that targeted the *IpxA* gene of thermotolerant campylobacter using a Firepol mastermix consisting of 200 µl of bovine serum albumen (BSA) at a concentration of 25mg/ml (Solis BioDyne), 50 µl (10 pmol) of each forward primer (*C. jejuni* CjejlpxAF ACAACTTGGTGACGATGTTGTA; *C. coli* ccollpxAF AGACAAATAAGAGAGAATCAG), 100 µl (10 pmol) reverse primer (CjejlpxAR CAATCATGDGCDATATGASAATAHGCCAT) (Eurofins) and 3200 µl of DNA free water (Klena et al., 2004).

DNA amplification was carried out in an Applied biosystems thermocycler using an initial denaturation step at 94°C for 5 minutes followed by 30 cycles of amplifications (denaturation at 94°C

for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min) ending with a final extension at 72°C for 10 minutes.

Those isolates that could not be characterised by this method were subjected to a 414 PCR which targets the *hipO* gene (personal communication NJ Williams, University of Liverpool). Briefly, using a Firepol mastermix consisting of 200 µl of BSA at a concentration of 25mg/ml, 50 µl (10 pmol) of forward primer (414F GGCGCTAAGGCAATGATAGA), 50 µl (10 pmol) reverse primer (414R TTGCAAAGCCACTACAAGCA) and 3300 µl of DNA free water. DNA amplification was carried out in an Applied biosystems thermocycler using an initial denaturation step at 94°C for 5 minutes followed by 30 cycles of amplifications (denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min)ending with a final extension at 72°C for 10 minutes.

MLST of whole genome of *C. jejuni* isolates

DNA extraction was performed by Trevor Jones at University of Liverpool. DNA was extracted from freshly grown *C. jejuni* using QIAamp DNA mini kit (QIAGEN) as per manufacturer's instructions.

Extracted DNA was sent to the Centre for Genomic Research (CGR) (University of Liverpool) where processing, sequencing and analysis of assemblages was carried out by Dr. Sam Haldenby. Briefly, shotgun libraries were prepared from normalised *Campylobacter* genomic DNA using TruSeq Nano Library Prep kits (Illumina inc.). Paired-end sequences (2x100 bp) of samples were multiplexed and run on an llumina HiSeq platform. Before analysing, assembled genomes were filtered, first by affinity to *C. jejuni* using BLAST query tool (NCBI) and then by size: assemblages smaller than 1.45 Mb and larger than 1.95 Mb were excluded from the analysis.

The assembled genomes were queried against the MLST database (http://pubmlst.org/campylobacter) to give a profile of the seven housekeeping genes , *aspA* (aspartase), *glnA* (glutamine synthetase), *gltA* (citrate synthase), *glyA* (serine hydroxymethyl transferase), *pgm* (phosphoglucomutase), *tkt* (transketolase), and *uncA* (ATP synthase alpha subunit) used to determine the sequence type. MLST alleles of STs not present in the database were assigned temporary allele IDs and STs and will be submitted to PubMLST.

4.3. Results

Birds

In total, 299 birds representing 25 species were captured between March 2015 and January 2016 (Table 4.1). Figure 4.3 shows how birds were trapped in the nets and then placed in paper bags for sample collection.

Approximately half the birds sampled were from the dairy farm (162, 54%) and half from the poultry farm (137, 46%). The most commonly sampled birds were blue tits (*Cyanistes caeruleus*, 31%), great tits (*Parus major*, 13%) and house sparrows (*Passer domesticus*, 10%) (Figure 4.4). Most birds were caught in spring (125, 42%) and winter (169, 57%), mainly because mid-summer trapping was severely affected by bad weather conditions.

Campylobacter isolates

The overall prevalence for *Campylobacter* species was 4.7% (CI 95% 2.8 to 7.7%), with fourteen isolates recovered from four species of birds, jay (*Garrulus glandarius*), dunnock (*Prunella modularis*), house sparrow (*Passer domesticus*) and rock dove / feral pigeon (*Columba livia*) (Table 4.2). Thirteen positive samples came from the dairy farm and one from the poultry farm (Figure 4.5). The majority of isolates were obtained between March and April 2015 (11/14) and the rest between November 2015 and February 2016 (3/14). All 14 isolates were *C. jejuni.* All boot sock samples were negative for *Campylobacter spp*.

Antimicrobial resistance

All fourteen isolates tested for antimicrobial resistance to ciprofloxacin, erythromycin and tetracycline were susceptible to ciprofloxacin and erythromycin. Only one isolate, from a dunnock sampled at the poultry farm, was resistant to tetracycline, the rest of the isolates were fully susceptible (Figure 4.6).

Figure 4.3 Mist nets A (i and ii): Birds being entangled. B: Birds being retrieved and put into bags. C: Feeder used to attract birds toward the nets, various birds can be observed trapped in the net behind the feeder.

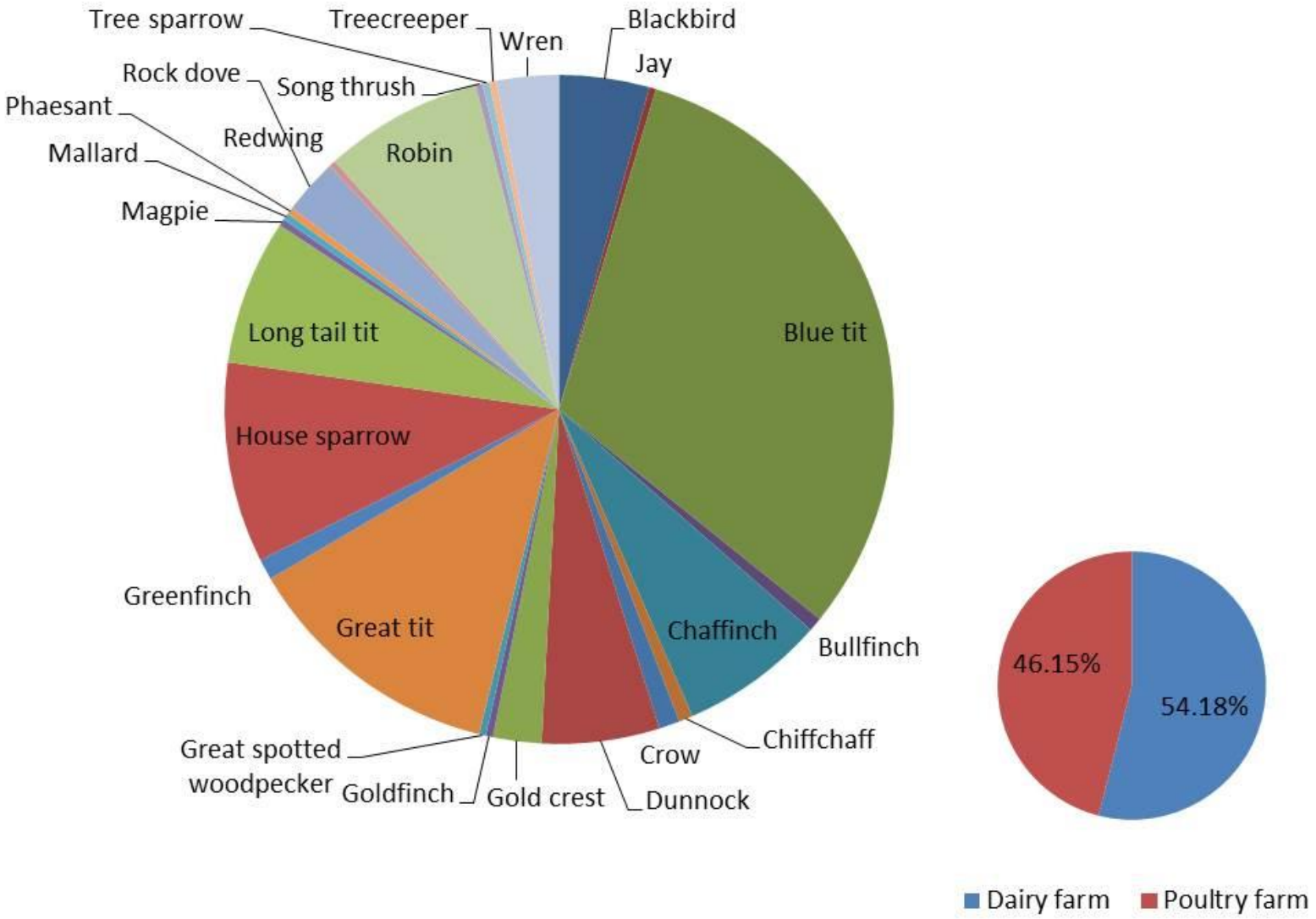

Figure 4.4 Proportion of birds trapped by species and farm.

Figure 4.5. Location of the *C. jejuni* positive birds from the dairy farm (A) and the poultry farm (B)

Figure 4.6. Antimicrobial susceptibility shown as areas without growth around the antimicrobial discs.

MLST diversity

All 14 isolates of *C. jejuni* were analysed using WGS and at the time of writing, 11 had their MLST profiles determined. Each of the eleven isolates had a distinct sequence type, two of which were novel (Table 4.2). All non-novel sequence types had previously been isolated from the dairy farm except that of the pigeon isolate (ST-2209, clonal complex 179). Novel STs and their associated alleles were compared to those in the PubMLST database. One novel ST was found to be related to those belonging to clonal complex 45 and the other novel ST to be most likely related to STs 436, 3401, 3402, 3413, 5321 and 5593. A full list of ST and the alleles isolated from the dairy and the poultry farms in this and previous studies is given in Appendix C.

Table 4.1. Species and number of sampled birds by location. *indicates those species from which *Campylobacter* was isolated.

Order	Family	Common name	Species	sampled	Dairy farm	Poultry farm
Passeriformes	Turdidae	Blackbird	*Turdus merula*	13	9	4
		Redwing	*Turdus iliacus*	1	1	0
		Song thrush	*Turdus philomelos*	1	0	1
	Corvidae	Jay*	*Garrulus glandarus*	1	1	0
		Crow	*Corvus corone*	3	0	3
		Magpie	*Pica pica*	1	0	1
	Paridae	Blue tit	*Cyanistes caeruleus*	93	34	59
		Great tit	*Parus major*	38	17	21
	Aegithalidae	Long tailed tit	*Aegithalos caudatus*	21	4	17
	Fringillidae	Goldfinch	*Carduelis carduelis*	1	0	1
		Chaffinch	*Fringilla coelebs*	21	18	3
		Bullfinch	*Pyrrhula pyrrhula*	2	0	2
		Greenfinch	*Carduelis chloris*	3	3	0
	Phylloscopidae	Chiffchaff	*Phylloscopus collybita*	2	0	2
	Prunellidae	Dunnock*	*Prunella modularis*	17	13	4
	Passeridae	House sparrow*	*Passer domesticus*	29	29	0
		Tree sparrow	*Passer montanus*	1	0	1
	Regulidae	Gold crest	*Regulus regulus*	7	4	3
	Muscicapidae	Robin	*Erithacus rubecula*	23	16	7
	Certhiidae	Treecreeper	*Certhia familiaris*	1	1	0
	Troglodytidae	Wren	*Troglodytes troglodytes*	9	2	7
Columbiformes	Columbidae	Rock dove / pigeon*	*Columba livia*	8	8	0
Galliformes	Phasianidae	Pheasant	*Phasianus colchicus*	1	0	1
Anseriformes	Anatidae	Mallard	*Anas platyrhynchos*	1	1	0
Piciformes	Picidae	Great spotted woodpecker	*Dendrocopos major*	1	1	0
		Total		299	162	137

Table 4.2. *Campylobacter* isolates and species found in birds from two farms in Cheshire. The overall prevalence of *Campylobacter* was 4.7% (14/299). All isolates were identified as *C. jejuni*

Species	Dairy farm			Poultry farm		
	C. jejuni	*ST*	*CC*	*C. jejuni*	*ST*	*CC*
Jay	1	Novel				
Dunnock	1	Novel				
	1	*267*	ST-283	1	*ND*	
House sparrow	1	21	ST-21			
	1	48	ST-48			
	1	267	ST-283			
	1	6985	ST-61			
	1	*ND*				
	1	*ND*				
Rock dove	1	220	ST-179			
	1	4447	ST-179			
	2	2209	ST-179			
total		13			1	
Prevalence (CI 95%)	8.02% (4.75 - 13.24%)			0.73% (0.13 - 4.02%)		
Overall prevalence (CI 95%)	4.68% (2.81 - 7.70%)					

ND. Not determined

4.1. Discussion

Campylobacter can infect a wide range of hosts, including humans; however clinical signs are much more common in humans than in most other species. A better understanding of the ecology of campylobacteriosis is important for developing control strategies for the disease in human populations. The purpose of the work described in this chapter was to determine the contribution of wild birds to the *Campylobacter* infections found in livestock and environment of two previously studied farms in Cheshire.

Previous studies have found *C. lari*, *C. coli* and *C. hyointestinalis,* as well as *C. jejuni* in wild birds in the Cheshire area (Brown et al., 2004, Hughes et al., 2009). However, in this study only *C. jejuni* was found in the 299 birds sampled. This may reflect the species range caught, and, in turn, the habitats sampled.

The overall prevalence of *C. jejuni* found in this study (5%) is similar to that found in other studies of wild birds in the UK, USA, New Zealand and Sweden (Craven et al., 2000, Waldenstrom et al., 2002, French et al., 2009, Keller et al., 2011, Sippy et al., 2012). The prevalence of campylobacteriosis in wild birds in the Cheshire area has been found to vary greatly: Hughes *et al*. (2009) found a low prevalence (1.4%), while Brown *et al*. (2004) reported a prevalence of 26% in bird faeces. However, Hughes *et al*. studied a much greater diversity of birds and over a wider geographic range, finding that between species prevalence ranged from 0-33%, while Brown *et al*. studied bird faeces collected from the ground in central Cheshire, thereby probably focussing on larger species such as corvids and gull (the latter not seen in fields and therefore not sampled in this study). Hughes *et al.* commented that 'prevalence estimates are likely to vary due to the use of different sampling regimens and culture methods', and differences in sampling regimens will obviously include the species (and populations) sampled. None of the STs found in this study were found in wild birds by Hughes *et al*. (2009).

The diversity of birds sampled appeared to reflect the bird species observed on the selected farms for this study. However, some species (eg corvids and pigeons) are numerically under-represented in the sampling compared to number of individuals seen. The most common species sampled (>20 individuals) were all small passerines: blue tits, great tits, house sparrows, robins, long tailed tits and chaffinches. Of these, *C. jejuni* was isolated only from house sparrows on the dairy farm, with a prevalence of 6/29 (21%). Of these small passerines, house sparrows and chaffinches are the main ground-feeding gramnivores – the other species eat largely insects. Furthermore, whereas

chaffinches were sampled from a range of sites, sparrows were sampled almost entirely around farm buildings. In this respect it is interesting that each positive sparrow was infected with a different ST.

This might suggest that the sparrows, despite living in close-knit flocks and with relatively small home ranges, do not have a host-adapted 'sparrow' ST, but rather become infected individually with campylobacter from their environment. Indeed, all the STs isolated from sparrows had previously been isolated from a range of sources on the farm. Dunnocks (17 birds) are mainly insectivores, similarly found in a variety of sites, particularly in hedgerows and trees, but are ground feeding. That 2/17 (at different farms) were infected with *C. jejuni*, but each bird with a different ST, may also suggest contamination rather than host-adapted ST. Thrushes (15 in total) eat mainly earthworms, insects and berries, and again were sampled mainly away from farm buildings, and were campylobacter-free in this study. Although Hughes et al found *C. jejuni* in both blackbirds and chaffinches, the prevalence was less than 5%, so unlikely to have been detected in this study owing to the relatively small numbers of individuals of these species caught.

If infection is related more to diet (and therefore exposure), then the two other groups of birds sampled that might be most expected to be infected with *C. jejuni* were corvids and pigeons, both of which are found feeding on the ground in and around animal buildings. Few corvids were sampled in this study, and only one, a jay, was positive. The prevalence among pigeons in this study (4/8) was rather higher than that found by Hughes et al (7/47 by isolation). Again, however, the four positive pigeons from the dairy farm, were infected with three different sequence types. Pigeons, of course, can range widely so would be exposed to infection at sites other than just the farm buildings. However, they tend also to flock. As for the house sparrows, the different STs suggest that infection in pigeons is not with a host-adapted ST but rather reflects environmental contamination.

This study took place in the context of a larger programme that sampled livestock and the environment on both farms over several years preceding this study (as yet unpublished). In that larger study, water, environment and animals (livestock) were sampled and a total of 91 different STs of *C jejuni* were identified (personal communication NJ Williams, University of Liverpool). All of the isolates obtained in this study matched those obtained in the larger environmental study with the exception of ST 2209 (from a pigeon) and the two novel sequences (from the jay and dunnock).

The *C. jejuni* STs found in these studies showed greater diversity among the water and environmental samples in these two farms (47 different STs from 175 environmental isolates and 55

different STs from 245 water isolates), while livestock seemed to have more host-specific STs and therefore less diversity with only 20 different STs identified from 290 isolates (Wedley, A. pers. Comm.)

Interestingly, all of the STs that coincided in both studies had previously `been isolated from water samples. *Campylobacter* isolated from water are often assumed to be from wildlife, in particular wild birds. We saw no evidence for that in this study – however, water fowl were rarely seen or tested. In another study conducted at the same time as this, wild rodents were trapped and sampled on the same farms. Only one animal, a field vole from the dairy farm, was positive for *C. jejuni.* This isolate has yet to be typed. So the ultimate source of these environmental campylobacter remains unknown.

One aim of this study was to determine whether or not any wildlife isolates of *C. jejuni* might be zoonotic. ST 2209 and ST 4447 have only previously been isolated from birds in the UK, Italy and Thailand (PubMLST, Bianchini et al., 2014b, Prachantasena et al., 2016). STs from this study previously isolated from wild birds and linked to human gastroenteritis are ST 21, ST 48, ST 220 and ST 267 (Colles et al., 2003, French et al., 2005, Kwan et al., 2008, Hughes et al., 2009, Griekspoor et al., 2013).

Generally, mild *Campylobacter* infections in people do not require treatment other than electrolyte replacement and rehydration, however, in severe or invasive human cases erythromycin, ciprofloxacin and tetracycline are the main antibiotics prescribed. (WHO, 2011). In 2010, in the EU, 52%, 2% and 21% of isolates from human cases were resistant to ciprofloxacin, erythromycin and tetracycline respectively, while isolates from broiler meat showed the highest rates of resistance at 50%, 2% and 22% to the same antibiotics (EFSA, 2012).

The fourteen isolates of *C. jejuni* found in this study were fully susceptible to ciprofloxacin, erythromycin and tetracycline, except for one isolate, from a dunnock, which was resistant to tetracycline. Other studies on wild bird have shown *C. jejuni* isolates with resistance to tetracycline, doxycycline, ciprofloxacin, norfloxacin and nalidixic acid (Chuma et al., 2000, Waldenstrom et al., 2005, Sippy et al., 2012). The lack of antibiotic resistance in the isolates from this study may be suggestive of colonisation of wild birds from sources where antibiotics are not widely used, as there has been suggested a link between antibiotic use in livestock and antibiotic resistance in isolates from other species like wild birds (Bonnedahl and Jarhult, 2014), however, this relationship seems to

be more important in waders and waterfowl (Veldman et al., 2013), which we did not sample in this study.

Table 4.3 Summary of ST and CC found in this study and previously described in in the UK (PubMLST database), EMIDA and ESEI study

Species	*C. jejuni*	*ST*	*CC*	Previously described sources (pubMLST database and EMIDA/ESEI studies)	Previously described human infection
Jay	1	*Novel*		N/A	no
Dunnock	1	Novel		N/A	no
	1	*267*	ST-283	Water, domestic animals, wild birds	yes
	1	*ND*		*N/A*	?
House sparrow	1	21	ST-21	Water, wild birds, livestock, domestic animals, environment	yes
	1	48	ST-48	Water, wild birds, livestock	yes
	1	267	ST-283	Water, domestic animals, wild bird	yes
	1	6985	ST-61	Water, environment, livestock, unknown source	no
	1	*ND*		*N/A*	*ND*
	1	*ND*		*N/A*	*ND*
Rock dove	1	220	ST-179	Water, wild birds, sand, environment	yes
	1	4447	ST-179	Water, poultry	no
	2	2209	ST-179	Wild birds	no

In this study, the majority of *C. jejuni* (79%) were isolated between week 11 and 14 (March 9th to April 5th 2015). Infection in humans is seen in spring and early summer (Nylen et al., 2002, Kovats et al., 2005, Louis et al., 2005, Meldrum et al., 2005), although in some countries there has been an increase in cases in winter, most likely associated with infection during travel particularly to warm places during winter months rather than local infection or an increase in "eating out" behaviour during Christmas months (Sopwith et al., 2006). In wild birds, summer warmer months have also shown an increase in prevalence of *Campylobacter* infection, except for starlings which can have a winter peak (Mohan, 2015) – this may be driven by starling behaviour, as starlings increasingly obtain much of their winter nutrition from agricultural and household waste disposal (Taitt, 2009). Thus the peak seen in this study was earlier than might be expected: this may have been due to an unusually mild winter and warmer spring (MetOffice, 2016).

Most of the wild bird isolates in this study were from an area on the dairy farm that is also used as a footpath for walkers and other domestic animals, such as horses and dogs. This location also used to be at the back of a slurry pit, which was emptied at the beginning of this year. Thus it might be

possible for birds to act as vectors of campylobacters between the farm and people. Environmental samples were taken using the boot sock method, but did not yield any *Campylobacter* isolates. While not enough samples were taken to determine the prevalence in the environment, the lack of positive samples from the environment together with the prevalence found among birds suggest that wild birds are not important contaminators of the environment.

Limitations of the study

The main limitations for this study were weather conditions for bird trapping and the abundance of birds at each farm. Even though both farms yielded approximately the same number of birds, success in trapping occurred during different seasons. Conclusions regarding the seasonal abundance of birds in each farm cannot be drawn based in this study as birds were sampled only for 12 months; a longer study would be needed to determine if the patterns of observed bird abundance are repeated each year or certain factors could have influenced the abundance in this particular year. As mentioned, certain weather characteristics can be an obstacle when using mist nets to trap birds; dampness and high wind can put caught birds at risk of injury or death and should be avoided when possible, in a similar way, trapping is limited to times when weather is not too cold and seasons when birds are not incubating eggs or younglings have just hatched (Redfern & Clark, 2001).

Wild birds are protected in the UK under various laws, including the Wildlife and countryside act of 1981; the presence of a licenced bird ringer from the RSPB allowed us to obtain non-invasively faecal samples at the same time as participating in the RSPB ringing scheme. This is an example of the positive outcomes of creating a network of people interested in the project. The network created for this project, albeit small, allowed us access, sampling, processing and genotyping of the samples obtained from the farms.

Overall this study, like those of Hughes et al (2009), Sippy et al. (2012), Craven et al (2000) and French et al. (2005) suggests that although wild birds have the potential to act as spreaders of *Campylobacter*, they are neither endemically infected nor likely to be significant contributors to the environmental loading of *Campylobacter*. Subtyping also demonstrated that the zoonotic potential of *Campylobacter* in wild birds is perhaps not as important as previously assumed, with many genotypes found 'exclusively' in birds as mentioned by French *et al*. (2005) and Griekspoor *et al.* (2013). Rather, wild birds appear to be net recipients of infection rather than sources. Indeed, some bird species (eg sparrows and pigeons) might be useful as sentinels for environmental

campylobacter contamination. Thus, although some of the STs found in wild birds correspond with those found in people, the wild birds in this study, at least, are unlikely to be either direct or indirect sources of human infection. Further studies will therefore be needed to identify the animal sources of *Campylobacter* within the farm environment and livestock.

5. General Discussion

The main aim of the work described in this thesis was to investigate the involvement of wildlife in four zoonoses in the UK. The four diseases studied were chosen based on their economic and/or public health impact: bovine tuberculosis, cryptosporidiosis, giardiasis and campylobacteriosis.

Wildlife has long been known, but also often assumed, to be the source of a wide range of zoonotic and livestock diseases, and in many cases regarded as reservoirs. Modern definitions of the term 'reservoir' have been discussed in Chapter 1, along with the need for if not agreed definitions, then at least an explanation of what is meant by the term in the context in which it is being used. Scientists, ecologists, policy makers and politicians often use 'reservoir' in vague and ambiguous ways, but whether consciously or not, do tend to use the term in the context of the population that they are interested in - the target population.

When planning control programmes or strategies, it is essential to start by defining the 'target population', for zoonotic diseases, this is normally humans, although in the case of bovine TB in a developed country such as the UK, where its impact on human health is no longer the main concern, cattle becomes the 'target population'.

Most recent attempts to pin down a definition of 'reservoir' (Haydon et al., 2002, Ashford, 2003, Cleaveland and Dye, 2009, Viana et al., 2014,) agree that one of its main characteristics is that it must be able to transmit the infection to the target population. However, such definitions can still cause confusion as many reservoirs are not a single species but multi-host systems where not necessarily all of the species/populations involved are essential to the transmission of infection to the target population (although together they form the maintenance community).

Furthermore, non-scientific literature or that produced for the general public often causes confusion by using vague taxonomic terms applied to non-target hosts. Such literature will often refer to 'wildlife' or 'rodents' or 'wild birds', without seemingly any recognition of the diversity of animals and their ecology. Several examples of apparent host-specificity of genotypes have been found in the studies described in this thesis.

It may, therefore be that the term ‘reservoir’ should be avoided, and terms such as maintenance populations and communities, and source populations, used instead. The issue is not simply one of definitions, but how the relaxed use of terms can allow conceptual misunderstanding – and how this in turn can hinder the development of effective and efficient control policies.

Even when the same agent is found in both target and potential source populations – and even when experimental studies show that infection or transmission between members of both populations is possible - this may not reflect what happens in nature. Some pathogens could be shared between host species but not transmitted between them. This has become clearer in recent decades with the use of high resolution, molecular characterisation methods, and, using these approaches provided evidence in the studies described in this thesis of host-adapted genotypes and of wildlife being most likely receivers rather than sources of infection.

Wildlife species have often been shown to share pathogens with humans (or other animals), and have subsequently been labelled ‘reservoirs’ and subjected to sometimes extreme methods of control, such as culling. For example, wild rodents and wild birds are often regarded as vermin and sources of disease and so shot or poisoned on farms. One specific example of this might be rats and other peridomestic rodents as sources of salmonellosis in livestock. A review of papers published on the role of rats in salmonellosis found that the vast majority reported the isolation of salmonellas from rats and mice as part of investigations of outbreaks in livestock, and then suggested that rats were the source of infection. However studies of wild rodents on British farms found salmonellosis to be extremely rare in wild rodents (M. Bennett pers comm). Himsworth et al. (2015), found in a study of salmonellosis in Canadian urban rats a prevalence of 0.5% and concluded that rats were ‘sponges’ acquiring salmonellas from their environments rather than being sources of infection.

Therefore, sometimes attempts to control disease in livestock by killing wildlife may be a waste of resources and yield no effective or long lasting results. It seems that an important limitation to developing disease control programmes is that of not really understanding the role of each host within a maintenance and source community, due in large part to the gaps in data available, particularly of wildlife infection dynamics (Nishiura et al., 2009).

The creation of networks that promote cooperation and provide different points of view and expertise can aid a project such as those presented in this thesis in many different aspects; from sample collection to genetic identification to public engagement.

Networks of academics, scientists and stakeholders were formed for each project. Perhaps the widest and most varied one was the one formed for the bTB in badgers project described in Chapter 2. As this project was being planned and developed, the involvement of not only veterinarians, both private and within the APHA, but of farmers and local conservation groups provided us with a unique insight into the thoughts and perceptions people outside the health trade had of the bTB epidemic in cattle, as well as what they believed was the role of badgers in it. Through the interaction and engagement of the different stakeholders we were able to collect and process the pre-determined number of carcasses and provide the parties involved with the results we obtained. This, however, was not to the liking of all parties involved. As one might suspect when working with several of stakeholder groups, each one had a preconceived idea of what the results of the study would be and in some cases, as expected, these ideas were not in agreement with each other and with what was found. This dissent, although sometimes heated, proved to not be a hindrance for the project, although it did cause some delays at times due to some stakeholders withdrawing their participation.

The networks developed for the studies covered in Chapters 3 and 4 involved a less diverse group of stakeholders, and possibly, in both cases, the target populations of the projects could be considered less controversial. Nevertheless, in both studies, the cooperation between wildlife groups, farmers and scientists was crucial for the development of the projects as well as their execution. This happened, however, not without some limitations, from delays in authorisations and health and safety approvals to allocation of resources towards the individual interests of the parties involved.

Nevertheless, in my experience, despite the potential obstacles and drawbacks of relying on a network of stakeholders, the advantages and benefits of working with a wide range of interested parties outbalance the downsides.

The overarching aim of the work described in this thesis, as set out in chapter 1 was to investigate, using four infectious diseases of economic and public health importance in the UK as models, the role of wildlife in the epidemiology of multi-host, zoonotic infections. The next section looks at each of these disease systems in turn.

5.1. *M. bovis* in badgers

The aim of this study was first to determine the feasibility of using road-killed badgers, and a stakeholder network, to investigate bTB in badgers at the edge of the English bovine TB epidemic in cattle.

As discussed in chapter 2, two studies, the first and larger study in the Edge county of Cheshire and the second smaller study in a 'Low Risk' area surrounding Stockport, were both successful at recruiting badger carcasses suitable for post mortem examination and the culture of *M. bovis*. The main potential bias in this method of sampling was possibly the differential engagement of stakeholders in the collection of carcasses, and future studies need to find ways of engaging more diverse contributors, for example pig, poultry, game and sheep farmers rather than just cattle farmers in such sampling. Nonetheless, the study demonstrated that this is a feasible approach, and, indeed, the approach has recently been adopted by Defra in order to study badgers in all 11 edge counties in England.

A second aim of this study was to assess whether or not bTB was present in Cheshire badgers, and the answer is that it is – indeed a prevalence of 21.3% is much higher than was originally expected and, perhaps more significantly, infected badgers were found from across Cheshire and not just near borders with higher risk counties. This estimated prevalence of bTB in road killed badgers is, furthermore, much higher than that found in surveys undertaken 25 - 40 years ago. However, the badger population has increased in the past decades (DEFRA, 2009): furthermore, the number of cattle breakdowns reported in Cheshire has also increased significantly in recent years (DEFRA, 2015b).

Until recently, cattle breakdowns in Cheshire were thought to be due to cattle bought from endemic areas, as indicated by the spoligotypes of *M. bovis* isolated and epidemiological tracing (Jahans and Worth, 2006). More recent isolates from cattle, however, and all the isolates from badgers in this study, were of spoligotype 25, which is the genotype found in neighbouring counties and seen as the regional spoligotype. That badgers shared the same spoligotype as most cattle in 2014, suggests that the spread of the epidemic in badgers is not due to spill over of non-regional spoligotypes from imported cattle, but rather is local expansion of either, or both, of the badger and cattle epidemics.

Unlike Goodchild *et al*. (2012) found in badgers in Wales, in the Cheshire study there was no correlation between cattle breakdowns and the probability of badgers being bTB positive in the

same area, unless more than 6 holdings had become OTFS or OTFW within a 5 km radius of a positive badger carcass. In Cheshire in 2014, new bTB testing protocols were introduced whereby all herds were tested annually, and radial testing was conducted around any herds testing positive. The increased number of cattle-positive holdings in 2014 could therefore be linked in part to the increased frequency of testing, rather than simply epidemic spread. The correlation found between cattle breakdowns and positive badger carcasses, but not until a relatively large number of farms had breakdowns within 5km, may indicate either that badger infection is driven more by cattle infection, or this might reflect farmers in areas where there have been cattle outbreaks being more engaged in submitting badger carcasses.

It was also interesting to find a high prevalence of bTB in badgers in the Stockport area, as this is regarded as a low risk area. If one conclusion from the Cheshire study is that bTB is more widespread in Cheshire than previously thought (so studies should be undertaken beyond the edge), then the Stockport study, albeit of a smaller number of badgers, is also suggestive that this is the case.

In addition to expanding the areas studied to the rest of the edge and beyond, further studies should also make more use of whole genome sequencing of badger and cattle isolates. Analyses of sequences, both phylogenetically and spatially, may help determine the drivers of the expansion of the epidemic in cattle, and answer the question vital to control policy of whether transmission is mainly in cattle or badgers.

Another interesting finding was that of the badger from North Wales infected with *M. microti*. Smith *et al*. (2009) suggested that vole TB might be a natural source of protection of badgers from *M. bovis*, and that if badgers are a significant source of cattle infection, then in areas with *M. microti* (such as North Wales, Cumbria and Northumberland) the bTB epidemic in cattle might slow or even halt. This is a further reason for undertaking studies of badgers in areas with historically low prevalence in cattle.

5.2. *Cryptosporidium* and *Giardia* in Llyn Cowlyd

The main aim of this study was to determine the role, if any, of wildlife in an annual peak in *Cryptosporidium* oocysts detected in a potable water reservoir, and, if oocysts were detected in wildlife and environmental samples, to determine the relationship of cryptosporidia from different sources to each other and to known genotypes, and to determine the likely zoonotic potential of cryptosporidia from these different sources. It was decided also to investigate the same samples for

Giardia, since cryptosporidia and *Giardia* are the two most common human infections associated with drinking water in the UK.

The main findings regarding *Cryptosporidium* oocysts found in the reservoir, were firstly the low prevalence of zoonotic *Cryptosporidium spp*. in the wild rodent population and perhaps more relevant, the high number of oocysts found to belong to non-zoonotic species of cryptosporidia in the water samples. For several years, DCWW has detected peaks in the number of *Cryptosporidium spp*. oocysts in water samples taken from Llyn Cowlyd, and it was important for DCWW to establish the source of the parasite in order to develop control strategies should these oocysts present a public health risk. Livestock, particularly sheep, were thought unlikely to be the source of these peaks for two main reasons: cryptosporidiosis is more prevalent in young animals (< 4 months) and lambing occurs earlier in the year (February to March) than the water peaks, and in theory, livestock are not allowed close to the shores of the reservoir. So the question arose as to whether the peak in oocysts in water was due to a wildlife source.

The main species of mammalian wildlife near the reservoir were small rodents and occasional insectivores. Species of rodents have been shown to harbour zoonotic species of *Cryptosporidium* particularly *C. parvum* and *C. ubiquitum* (Chalmers et al., 1997, Torres et al., 2000,Perec-Matysiak et al., 2015). However, the prevalence of zoonotic cryptosporidia at LLyn Cowlyd was very low (3/97) in rodents – rather they were infected with largely novel genotypes, itself an interesting finding. *C. parvum* and *C ubiquitum* were found in wood mice, however it is not possible to determine if wood mice are sources of contamination or merely reflecting environmental contamination (although it may be relevant that other rodent species, equally exposed to environmental contamination were not shedding either species of *Cryptosporidium*). Water samples taken from feeder streams showed a higher number of *C. ubiquitum,* which is zoonotic, than raw water samples, which may simply reflect dilution, as well as a number of novel genotypes. Raw water samples showed small numbers of potentially zoonotic cryptosporidia, but the majority of PCR positive samples contained yet another novel genotype not present in any other samples, including the feeder streams.

Livestock was observed every day near the shores of the reservoir and therefore should be regarded as potential sources of contamination. Unfortunately, samples from livestock (the sampling plan agreed was only to collect livestock faeces from transects) were few and so no firm conclusions can be drawn regarding the role they play in the zoonotic oocysts found in water. However – as the

zoonotic cryptosporidia found in water have in the past been associated with livestock, it would be prudent to improve the separation of these animals from the lake.

Without further investigation, one hypothesis is that fish and birds might be associated with the *Cryptosporidium* peaks found in water, particularly fish as the most common novel genotype found in raw water was not found in feeder streams. However, as novel genotypes (ie never reported in people) and as no infection in humans has been linked to fish or bird-specific genotypes (Fayer, 2010, Ryan, 2010), they are unlikely to be zoonotic, and in terms of public health, water from Llyn Cowlyd can probably be regarded as very low risk.

The sequencing of *Giardia* detected in this study suggested a similar story to that of *Cryptosporidium:* all the rodent samples contained a novel assemblage and the livestock samples and most of the water samples contained only assemblage E which is non-zoonotic and found mainly in ruminants. The zoonotic assemblage A was found in three water samples: sources could include humans, dogs and livestock, this last one being the most likely source due to the abundance of sheep observed in the area.

What was clear in both cases, was that while traditional approaches of detecting (oo)cysts might have indicated an important role for wildlife in the contamination of water with zoonotic protozoa, sequencing approaches suggest that wildlife were largely infected with novel and host-adapted genotypes and therefore had small, if any, zoonotic risk associated with drinking the water. Most likely, livestock was the main source of small numbers of zoonotic (oo)cysts found in water, and while the source of the annual peaks in cryptosporidia in Llyn Cowlyd remains undetermined, the oocysts are unlikely to be zoonotic.

Further studies aimed at identifying the sources of water contamination would entail far more extensive sampling and sequencing of larger regions of the genomes of both protozoa in order to build better phylogenies that might correlate with hosts.

5.3. *Campylobacter* in wild birds

The aim of this study was to determine the rates of *Campylobacter* spp. infection of wild birds on a poultry and a dairy farm, and if found, to genotype the isolates and investigate the potential role of wild birds in the epidemiology of on-farm campylobacter transmission.

The low prevalence (14/299) of *C. jejuni* found in wild birds in the two farms where the study took place, might suggest a low risk of infection from wild birds to livestock and humans. Similar prevalences have also been found in wild birds in other studies (Craven et al., 2000, French et al., 2009, Sippy et al. 2012).

Most infection was found in a small number of species, corvids, house sparrows and pigeons, of which corvids need to be investigated further since only very few of these were sampled. However genotyping (STs) demonstrated that not all genotypes have been associated with human disease, and that almost every individual infected bird had a different genotype of *C. jejuni*. What's more, while these genotypes had previously been found on these farms in environmental (particularly water) samples, none of them were found in a previous study of wild birds in Cheshire (Hughes et al. 2009). All of this suggests that wild birds – or at least the species sampled in this study – are more likely to be sponges than sources of infection with campylobacters in farm environments.

5.4. Conclusions and final remarks

This chapter began with a discussion of some of the concepts and language around cross-species transmission and in particular zoonoses, and then summarised some of the outcomes of each of the individual studies that form the core of this thesis.

There are some common themes arising out of each of the chapters, the first of which is that of evidence versus assumptions, and how these issues apply particularly to wildlife infection and disease. The badger work arose out of different stakeholder groups arguing about the role of badgers in the expanding bTB epidemic in cattle. Some stakeholders argued that the expansion of bTB into Cheshire must be due to badgers, and others equally vehemently that it must be poor farming practices. Yet there was no evidence as to whether or not bTB was present in any badgers in Cheshire. Enthusiastic stakeholder engagement had both advantages and disadvantages, but overall provided an incredibly rewarding means of doing research. The study not only answered the question it set out to answer – namely is there bTB in badgers in Cheshire? - but raised some interesting questions about drivers of the expanding bTB epidemic in cattle and badgers and the amount of cross species transmission that might be occurring. It may even be that some of those questions about drivers of the epidemic will be answered in the larger-scale study of edge counties throughout England, established largely through the success of this stakeholder-led, feasibility study (http://www.nottingham.ac.uk/vet/survey-for-tb-in-road-killed-badgers.aspx, http://www.surreyvetpathology.com/pathology-services/defra-funded-badger-tb-survey). Further

answers to questions about who-infects-whom may also come from planned sequencing studies on isolates archived from the Cheshire study, as higher resolution differentiation of isolates is needed to accompany the spatial data also archived than can be gained by simply spoligotyping.

While the role of wildlife as sources or receivers of bTB has not been determined in this study, stronger indications of the role of wildlife in giardiasis, cryptosporidiosis and campylobacteriosis have been gained – largely through the use of sequence-based characterisation of the pathogens from different hosts and environments. For both protozoa, and for campylobacter, traditional approaches would have suggested wildlife as sources of infection to livestock and/or humans. This might in turn have led to expensive and unsuccessful control programmes – for controlling disease in wild populations is notoriously difficult – or to decisions not to intervene owing to the magnitude of the task. By focussing research on the evidence gaps that need filling in order to develop control measures, rather than assuming knowledge or studying larger ecological/epidemiological questions, some practical outcomes have been achieved in the studies described in this thesis. Thus while the source(s) of the annual peaks in *Cryptosporidium* oocysts seen in raw water at Llyn Cowlyd have not been identified, it seems unlikely that these oocysts are a risk to public health and the little evidence of zoonotic contamination of the water should be readily controlled by increased biosecurity – removing access of livestock to the edges of the lake. Similarly, wild birds – albeit that more wild bird species should be investigated – appear to be indicators of environmental campylobacter rather than sources of it, and need not be the focus of control programmes on farms.

Of course many interesting, and some important, questions remain. Ecological questions include the relationship between host and parasite phylogenies, the barriers to cross-species transmission (physiological, immunological or simply behavioural and contact-based). The important questions that need answering for bTB are determining the drivers of the expansion of the epidemic. However, even for TB, the involvement of stakeholders in the Cheshire study, and frequent communication of results and progress, means that these stakeholders at least understand the issues involved in getting the answers they so want – and the approach taken has helped empower those stakeholders to demand those answers.

'One health' as mentioned in Chapter 1, has many different definitions, and is an approach claimed by many different institutions. Probably the best summary of the One Health approach is that of the Manhattan Principles, that emerged from a meeting brought together in 2004 by the Wildlife Conservation Society (WCS, 2004), and which state amongst other things that:

‘ …civil society, the global health community, and institutions of science … holistically approach the prevention of epidemic/epizootic disease and the maintenance of ecosystem integrity by… recognizing the link between human, domestic animal, and wildlife health, and the threat disease poses to people, their food supplies and economies, and the biodiversity essential to maintaining the healthy environments and functioning ecosystems we all require.’

According to this, the key to achieving a true ‘one health’ is not simply interdisciplinary research, but interdisciplinary research applied to solving problems taking into account the welfare of animals as well as the people whose lives are affected by not just the disease but the methods taken to control it.

The studies described in this thesis have been collaborative and interdisciplinary – they have involved working with ecologists, microbiologists, veterinarians, epidemiologists, medics and statisticians. But perhaps most importantly they have involved working with a range of stakeholders – farmers, conservationists, government and utility companies. The involvement of so many partners also meant that each one had their own particular interest in mind, which perhaps at times, put extra constraints on what might be done, and so the studies raise as many questions as they answer. Nevertheless, in the end, it was encouraging to see a ‘one health’ approach reached through the cooperation between all the parts that made this project possible.

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Appendix A

Llyn Cowlyd trapping scheme

Day/week	Time	1	2	3
Monday	Morning (7:00 – 8:00)	Prepare safety kit Ensure sat phone is charged Prepare sampling equipment (bags, bootsocks, etc.) Prepare traps, feed, bedding	Prepare safety kit Ensure sat phone is charged Prepare sampling equipment (bags, bootsocks, etc.) Prepare traps, feed, bedding	Prepare safety kit Ensure sat phone is charged Prepare sampling equipment (bags, bootsocks, etc.) Prepare traps, feed, bedding
	Morning (8:00 – 12:00)	Arrive to LC Set up traps on grid **1** and **2** (50 traps each) on a 50x50m grids	Arrive to LC Set up traps on grid **3** and **4** (50 traps each) on a 50x50m grids	Arrive to LC Set up traps on grid **5** and **6** (50 traps each) on a 50x50m grids
Tuesday	Morning (7:00 – 11:00)	Arrive to LC (8:00) Check and reset traps Identify, record and mark rodents Release rodents Collect faeces from trap Clean traps with ethanol and replace bedding and feed Reset traps	Arrive to LC (8:00) Check and reset traps Identify, record and mark rodents Release rodents Collect faeces from trap Clean traps with ethanol and replace bedding and feed Reset traps	Arrive to LC (8:00) Check and reset traps Identify, record and mark rodents Release rodents Collect faeces from trap Clean traps with ethanol and replace bedding and feed Reset traps
	Afternoon (12:00 – 17:00)	Back at Leahurst process, label and store samples (4°C) Check trapping record	Back at Leahurst process, label and store samples (4°C) Check trapping record	Back at Leahurst process, label and store samples (4°C) Check trapping record
Wednesday	Morning (7:00 – 11:00)	Arrive to LC (8:00) Check and reset traps Identify, record and mark rodents Release rodents Collect faeces from trap Clean traps with ethanol and replace bedding and feed Reset traps	Arrive to LC (8:00) Check and reset traps Identify, record and mark rodents Release rodents Collect faeces from trap Clean traps with ethanol and replace bedding and feed Reset traps	Arrive to LC (8:00) Check and reset traps Identify, record and mark rodents Release rodents Collect faeces from trap Clean traps with ethanol and replace bedding and feed Reset traps
	Afternoon (12:00 – 17:00)	Back at Leahurst process, label and store samples (4°C) Check trapping record	Back at Leahurst process, label and store samples (4°C) Check trapping record	Back at Leahurst process, label and store samples (4°C) Check trapping record
Thursday	Morning (7:00 – 11:00)	Arrive to LC (8:00) Check and collect traps Identify, record and mark rodents Release rodents Collect faeces from trap Collect traps	Arrive to LC (8:00) Check and collect traps Identify, record and mark rodents Release rodents Collect faeces from trap Collect traps	Arrive to LC (8:00) Check and collect traps Identify, record and mark rodents Release rodents Collect faeces from trap Collect traps
	Afternoon (12:00 – 17:00)	Back at Leahurst process, label and store samples (4°C) Check trapping record	Back at Leahurst process, label and store samples (4°C) Check trapping record	Back at Leahurst process, label and store samples (4°C) Check trapping record
Friday	Morning (8:00 – 11:00)	At Leahurst, autoclave traps, feed and bedding	At Leahurst, autoclave traps, feed and bedding	At Leahurst, clean traps
	Afternoon (12:00 – 17:00)	Check and prepare consumables (kits) for next week	Check and prepare consumables (kits) for next week	End of trapping weeks

Appendix B

Qiagen DNA mini kit (*M. bovis*)

1. Re suspend 3-4 colonies in 180 µl of **Buffer ATL**
2. Add 20 µl of **proteinase K**
3. Vortex vigorously
4. Incubate at 56°C for 3 hours (vortex occasionally)
5. Add 200 µl of **Buffer AL**. vortex the sample
6. Incubate at 70°C for 10 mins
7. Add 200 µl of **ethanol**. Vortex
8. Place a Qiamp column in a collection tube
9. Add the sample to the column
10. Centrifuge for 1 min (6,000 x g)
11. Discard collection tube and place column in new collection tube
12. Add 500 µl of **Buffer AW1**
13. Centrifuge for 1 min (6,000 x g)
14. Discard collection tube. Place column in new collection tube
15. Add 500 µl of **Buffer AW2**
16. Centrifuge for 3 mins (14,000 rpm or full speed)
17. Place column in new collection tube and centrifuge at full speed for 1 min
18. Discard collection tube and place column in 2ml Eppendorf tube
19. Add 200 µl of **distilled water**
20. Incubate at room temperature for 5 mins
21. Centrifuge for 1 min (6,000 x g)
22. Repeat steps 19-21 to ensure elution of all DNA

Steps 1 to 4 should be done inside the CL3 facility

Modified Macherey-Nagel DNA Extraction Method for DNA extraction from *Cryptosporidium spp.* oocysts from cattle and sheep faecal Samples

1. Centrifuge sample in 50ml falcon tube at 2750rpm for 10 mins and discard supernatant.
2. Add 1ml TE buffer (5ml 1M TrisHCL, 1ml 0.5M EDTA, 494ml dH_2O; pH 8) and resuspend by vortexing vigorously for 30s.
3. Centrifuge sample in 50ml falcon tube at 2750rpm for 10 mins and discard supernatant
4. Resuspend pellet in 200ml Buffer T1 and vortex vigorously. Transfer to 1.5ml Eppendorf.
5. 10 Freeze/Thaw cycles in Liquid Nitrogen.
6. Add 25µl Proteinase K and vortex to mix.
7. Incubate overnight at 56^{o}C.
8. Vortex the samples vigorously and incubate at 95 ^{o}C for 10 mins.
9. Vortex the samples.
10. Add 200µl Buffer B3, vortex vigorously and incubate at 70^{o}C for 10 mins. Vortex briefly to mix the samples.
11. Centrifuge at 11,000 x g for 5 mins to remove insoluble particles and transfer the supernatant to a new Eppendorf.
12. Add 210µl EtOH (100%) and vortex vigorously.
13. Place a spin column in a collection tube and add the whole sample.
14. Centrifuge for 1 min at 11,000xg. Discard flow through and replace column into collection tube (repeat centrifuge if some of the sample is retained in the column).
15. Add 500µl Buffer BW and centrifuge at 11,000xg for 1 min. Discard flow through and replace column into collection tube.
16. Add 600µl Buffer B5 and centrifuge at 11,000xg for 1 min. Discard flow through and replace column into collection tube.
17. Centrifuge column at 11,000xg for 1 min to dry the membrane.
18. Place column into a 1.5ml Eppendorf and add 100µl UP water. Spin at 11,000 x g for 1 min. to elute DNA.

Fast Stool Mini Kit (Qiagen)

1. Weight the stool sample
2. Add 10 vol of **InhibitEx Buffer** (10ml per 1g stool
3. Vortex for 1min
4. Pipette 2ml in a Eppendorf tube
5. Incubate for 10 mins at 95°C
6. Allow to cool at room temperature
7. Vortex for 15 sec and centrifuge at 20,000 rcf for 1 min
8. Pipette 15 µl **Proteinase K** in a new Eppendorf
9. Add 200 µl of supernatant
10. Add 200 µl of **AL** buffer
11. Vortex 15 sec
12. Incubate at 70°C for 10 min
13. Centrifuge briefly
14. Add 200 µl of **ethanol** (96-100%)
15. Vortex
16. Centrifuge briefly
17. Label the lid of **a QIAmp spin column** and place in a 2ml collection tube
18. Pour the complete lysate
19. Centrifuge at 20,000 rcf for 1 min
20. Repeat if the lysate has not passed completely through the column
21. Place the spin column in a new collection tube.
22. Discard the tube with the filtrate
23. Add 500µl of **AW1 buffer**
24. Centrifuge at 20,000 rcf for 1 min
25. Place the spin column in a new collection tube.
26. Discard the tube with the filtrate
27. Add 500µl of **AW2 buffer**
28. Centrifuge at 20,000 rcf for 4 min
29. Transfer the column into a new 1.5 Eppendorf
30. Discard the tube with the filtrate.
31. Add 100 µl of **ATE buffer** directly to the membrane
32. Close the cap. Incubate at room temperature for 5 min
33. Centrifuge at 20,000 rcf for 1 min to elute DNA

CHELEX DNA extraction

1. To an Eppendorf containing 300 µl of chelex, add 2-3 isolated colonies.
2. Incubate at 95°C for 10 minutes.
3. Spin tube for 2 minutes at 13000 rpm.
4. Add 50 µl of the supernatant to 450 µl of sterile distilled water.

Appendix C

Cheshire badgers location, age, sex, weight and bTB results

BADGER NUMBER	DATE OF SUBMISSION	SH cat	LOCATION	SEX	AGE GROUP	WEIGHT	bTB RESULTS
1	13/02/2014	V	CONGLETON	F	A	10.7	+
2	17/02/2014	V	AUDLEM	M	A	11	
3	18/02/2014	F	POULTON	M	A	9.5	
4	20/02/2014	V	COMBERMERE	F	Y	9.6	
5	25/02/2014	F	WHITCHURCH	M	A	9.8	
6	22/02/2014	O	WINSFORD	F	Y	7.9	
7	24/02/2014	F	FARNDON	M	A	11	
8	24/02/2014	F	FARNDON	M	Y	11	
9	26/02/2014	O	BOLLINGTON	M	Y	10.5	+
10	27/02/2014	F	HIGHER WHITLEY	M	Y	11	
11	04/03/2014	V	CREWE	M	A	11.5	
12	04/03/2014	V	WHITCHURCH	O	A	11	
13	04/03/2014	F	NORTHWICH	M	A	11	
14	06/03/2014	O	MACCLESFIELD	F	A	11	
15	10/03/2014	F	FARNDON	F	A	10	
16	10/03/2014	F	KNUTSFORD	F	A	12	+
17	16/03/2014	F	NANTWICH	F	Y	8.5	+
18	24/03/2014	F	NORTHWICH	M	A	10	+
19	27/03/2014	V	RIDLEY	M	A	10	
20	27/03/2014	V	ALPRAHAM	M	Y	9.4	
21	27/03/2014	V	HANKELOW	F	Y	9.5	
22	27/03/2014	V	WHITCHURCH	F	A	12	
24	01/04/2014	V	AUDLEM	F	A	11	+
25	02/04/2014	O	TARPORLEY	M	A	11.5	
26	10/04/2014	V	CLOTTON	M	Y	7	
28	14/04/2014	F	LOWER PEOVER	M	A	8.5	
29	16/04/2014	V	NANTWICH	F	A	10	+
30	16/04/2014	F	CONGLETON	F	A	8	+
31	17/04/2014	F	MACCLESFIELD	M	Y	11	
32	21/04/2014	W	NANTWICH	F	Y	4.5	
33	28/04/2014	V	FRODSHAM	F	A	9	
34	29/04/2014	W	MALPAS	M	A	11	
35	30/04/2014	V	AUDLEM	F	A	9	
36	30/04/2014	V	TARPORLEY	M	A	11	
37	30/04/2014	F	NORTHWHICH	F	A	8.5	
39	02/05/2014	F	KNUTSFORD	F	Y	11	
40	06/05/2014	F	KNUTSFORD	F	A	11	
41	07/05/2014	F	DELAMERE	M	A	7.8	

Cont.							
42	09/05/2014	F	NANTWICH	M	A	10	
43	15/05/2014	V	TARPORLEY	M	A	12	
45	27/05/2014	F	KNUTSFORD	F	A	9.5	
46	28/05/2014	V	KELSALL	M	A	9	
47	31/05/2014	V	CREWE	M	A	7	+
48	11/06/2014	F	CHESTER	F	Y	10	
49	12/06/2014	F	TARPORLEY	F	Y	8	+
51	16/06/2014	V	KNUTSFORD	F	A	11	
52	16/06/2014	F	KNUTSFORD	F	A	8.5	
53	23/06/2014	F	LITTLE BUDWORTH	M	A	9.5	
54	27/06/2014	F	TARPORLEY	F	Y	7.5	
55	03/07/2014	F	TARPORLEY	F	Y	6	
56	05/07/2014	F	MALPAS	M	A	8	
57	08/07/2014	W	TARPORLEY	M	A	4.5	
58	03/08/2014	F	GRINDLEY BROOK	M	A	9	
59	06/08/2014	O	KNUTSFORD	M	Y	11.5	
60	07/08/2014	F	POULTON	M	A	10	
61	08/08/2014	F	CONGLETON	M	A	4	+
62	16/08/2014	V	CONGLETON	F	A	9.5	+
63	23/08/2014	V	WYNBURY	M	A	12	
64	01/09/2014	O	MACCLESFIELD	F	Y	7.5	
65	03/09/2014	F	KNUTSFORD	F	A	11	
66	03/09/2014	F	CONGLETON	M	Y	9.5	
67	07/09/2014	F	CONGLETON	F	A	10	+
68	09/09/2014	O	CHESTER	M	Y	10.5	
69	09/09/2014	V	AUDLEM	F	Y	11	
70	11/09/2014	F	TARPORLEY	F	Y	7	
71	11/09/2014	O	CREWE	F	Y	8	
72	12/09/2014	O	CREWE	F	Y	8	
73	18/09/2014	W	STOKE ON TRENT	F	A	9	
74	19/09/2014	V	MACCLESFIELD	M	A	13	
75	19/09/2014	V	CONGLETON	F	Y	12	+
76	23/09/2014	O	CHESTER	F	A	9	
77	24/09/2014	V	AUDLEM	M	A	10	
78	25/09/2014	O	KNUTSFORD	F	Y	14.5	
79	26/09/2014	W	MACCLESFIELD	M	A	13	+
80	02/10/2014	F	KNUTSFORD	M	A	16	
81	02/10/2014	V	NORTHWICH	M	A	9.5	+
82	12/10/2014	O	WARRINGTON	M	A	13.5	
83	13/10/2014	F	CHESTER	M	A	13	
84	22/10/2014	F	KNUTSFORD	M	A	18	
85	24/10/2014	V	NORTHWICH	O	Y	13.5	
86	28/10/2014	O	CHELFORD	M	Y	9.5	+

Cont.							
87	06/11/2014	V	NANTWICH	M	Y	13.5	
88	11/11/2014	V	NORTHWICH	M	A	12	
89	11/11/2014	V	FRODSHAM	M	A	14	
90	13/11/2014	O	CREWE	F	A	15	+
91	14/11/2014	V	NANTWICH	F	Y	11	
92	18/11/2014	V	MACCLESFIELD	M	A	13.5	
93	19/11/2014	F	FRODSHAM	M	A	15	
95	09/12/2014	F	MACCLESFIELD	F	A	6	
96	09/12/2014	F	KNUTSFORD	F	A	14	+
97	20/01/2015	V	NANTWICH	M	Y	12.5	
98	20/01/2015	F	CHESTER	F	Y	8.5	
99	23/01/2015	W	CREWE	M	A	10	
102	29/01/2015	V	FRODSHAM	M	A	12.5	+

SH cat. V:veterinarian, F: farmer, W: wildlife or rescue group, O: other

Cheshire positive badgers details

Badger number	Sex		Age		Positive pool						total
	M	F	Young (<1yr)	Adult (>1 yrs)	Head	Thorax	Lungs	Abd.	Carcass	Other	
001		1		1		1					1
009	1		1						1		1
016		1		1		1					1
017		1	1					1			1
018	1			1		1					1
024		1		1					1		1
029		1		1	1	1					2
030		1		1					1		1
047	1			1	1	1	1	1	1		5
049		1	1		1						1
061	1			1	1	1		1	1		4
062		1		1	1			1	1		3
067		1		1	1				1		2
075		1	1		1				1		2
079	1			1	1				1		2
081	1			1	1				1		2
086	1		1					1			1
090		1		1	1				1		2
096		1		1	1				1		2
102	1			1	1						1
TOTAL	8	12	5	15	12	6	1	5	12	0	36

Badgers submitted from beyond of Cheshire

Badger ID	sex	age	location	provenance	bTB result
23	f	a	Shropshire	RSPCA	no
27	f	y	Staffordshire	RSPCA	no
38	f	y	North Wales	road kill	+
44	f	a	West Midlands	RSPCA	no
50	m	y	Shropshire	road kill	no
94	m	y	Derbyshire	road kill	no
100	m	a(o)	Shropshire	RSPCA	+
101	f	a	Staffordshire	RSPCA	+
M01	f	a	Stockport	road kill	no
M02	f	a	Stockport	road kill	no
M03	f	a	Stockport	road kill	no
M04	f	a	Stockport	road kill	no
M05	f	a	Stockport	road kill	+
M06	m	a	Stockport	road kill	no
M07	m	a	Stockport	road kill	+
M08	m	a	Stockport	road kill	no
M09	m	a	Stockport	road kill	no
M10	m	a(o)	Stockport	road kill	no
M11	m	a	Stockport	road kill	no
M12	o	a	Stockport	road kill	no
M13	m	a	Stockport	road kill	no
M14	f	a	Stockport	road kill	no
M15	f	a	Stockport	road kill	no
M16	m	a	Stockport	road kill	no
M17	o	a	Stockport	road kill	no
M18	f	a	Stockport	road kill	no
M19	o	a	Stockport	road kill	no
M20	f	a	Stockport	road kill	+
M21	m	a	Stockport	road kill	no
M22	m	a	Stockport	road kill	+
M23	f	a	Stockport	road kill	no
M24	f	a	Stockport	road kill	no
M25	f	a	Stockport	road kill	+
M26	f	a	Stockport	road kill	+
M27	m	a	Stockport	road kill	+
M28	o	a	Stockport	road kill	+
M29	f	a	Stockport	road kill	no
M30	f	a	Stockport	road kill	no

Campylobacter jejuni isolates found in the dairy and poultry farms in previous (ESEI, EMIDA) and in this study

ST	ST - complex	Dairy farm			Poultry farm			Disease in humans	isolation from other sources (PubMLST)
		Previous study	Sample origin	Current study	Previous study	Sample origin	Current study		
5	353				✓	E,BS,W		gastroenteritis	chicken (o/m)
11	445	✓	E,W					gastroenteritis	chicken (o/m), cattle (carrier)
19	21	✓	E,F,W					systemic disease, gastroenteritis	Sheep (o/m), chicken (o/m), lamb (o/m), duck (o/m), cattle, goat.
21	21	✓	E,F,W	✓	✓	E		gastroenteritis	Cow (milk), lamb (o/m), beef (o/m), chicken (o/m), cattle, dog, duck, wild bird, environmental water, other animal.
22	22	✓	E					gastroenteritis	Lamb (o/m), farm slurry, chicken (o/m), cattle, sheep, dog, other animal.
38	48	✓	F					systemic disease, gastroenteritis	Sheep, chicken (o/m), environmental waters, cattle, starling.
42	42	✓	E,F,W		✓	E		gastroenteritis	Cow (milk), lamb (o/m), cattle, sheep, chicken (o/m), duck, wild bird, starling, environmental water, other animal.
45	45	✓	E,F,W					systemic disease, gastroenteritis	Cattle, chicken (o/m), beef (o/m), lamb (o/m), turkey, chick, sheep, other animal, environmental waters, soil, wild bird, starling, duck, dog.
48	48	✓	E,F,W	✓	✓	W		systemic disease, gastroenteritis	Sand (bathing beach), lamb (o/m), beef (o/m), calf, cattle, lamb, chicken (o/m), sheep, other animal, farm environment.
50	21	✓	E		✓	E,BS		systemic disease, gastroenteritis	Drinking water, chicken (o/m), lamb (o/m), beef (o/m), sheep, cattle, other animal.
51	443	✓	E,F,W		✓	E,BBS		gastroenteritis	Chicken (o/m), duck.
52	52	✓	E,W					systemic disease, gastroenteritis	Sheep, lamb (o/m), chicken (o/m), cattle.
53	21	✓	F					systemic disease, gastroenteritis	Calf, chick, starling, cattle, wild bird, other animal, sheep, chicken (o/m).
61	61	✓	F					systemic disease, gastroenteritis	Lamb (o/m), chicken (o/m), beef (o/m), cattle, giraffe, sheep, environmental waters, other animal.

Cont.

122	206	✓	F		✓	E,W		systemic disease, gastroenteritis	chicken
132	508	✓	E,F,W					gastroenteritis	Chicken (o/m), dog.
137	45	✓	E,F,W		✓	E,F,W		gastroenteritis	Starling, chicken (o/m), wild bird, sheep, other animal, goose, cattle, dog.
177	177				✓	E		gastroenteritis	starling, sand (bathing beach)
220	179	✓	E,W	✓				gastroenteritis	starling, sand (bathing beach)
257	257	✓	F,W		✓	E,BBS,W		gastroenteritis	Chicken (o/m), turkey, starling, sheep, cattle, dog, farm environment.
262	21	✓	E,W					systemic disease, gastroenteritis	beef (o/m), broiler environment, cattle, chicken (o/m), farm environment, farm slurry, lamb (o/m), pork (o/m), sheep
267	283	✓	W	✓	✓			gastroenteritis	Cattle, chicken (o/m), dog, drinking water, other animal, sheep, starling.
270	403	✓	E,F,W			E,BBS		gastroenteritis	Beef (o/m), cattle, lamb (o/m), pig, sheep.
273	206	✓	E					gastroenteritis	Beef (o/m) cattle, chicken (o/m), dog, sheep.
403	403	✓	E,W					systemic disease, gastroenteritis	Dog, pig.
448		✓	E,W		✓	E		gastroenteritis	Wild bird, other animal.
508	508	✓	NA					gastroenteritis	Dog, wild bird.
520	21	✓	E,F,W					gastroenteritis	Sheep, cattle.
573	573	✓	E,F,W		✓	E,F,BBS,W		gastroenteritis	chicken (o/m)
677	677	✓	E					systemic disease, gastroenteritis	Cattle, chicken (o/m), environmental waters, other animal, rabbit, starling, wild bird.
682	682	✓	E					gastroenteritis	Starling.
683		✓	W						Starling.
693		✓	W						Goose.
794	677	✓	E,W					gastroenteritis	Wild bird.
799	952	✓	E,W						Environmental waters.

Cont.

827	828	✓	E,F,W					gastroenteritis	Cattle, chicken (o/m), cow (milk), duck, environmental waters, soil, other animals, lamb (o/m), farm environment, sheep.
828	828	✓	E		✓	E		gastroenteritis	chicken (o/m), environmental waters, lamb (o/m)
845	45	✓	W					gastroenteritis	chicken (o/m)
945	1287	✓	E					gastroenteritis	chicken (o/m), environmental waters, duck
952	952	✓	E,W						rabbit
990	257	✓	F					gastroenteritis	other animal
995		✓	W						Environmental waters.
1044	658	✓	E,F,W					gastroenteritis	chicken, dog
1231	1275	✓	W		✓	E		gastroenteritis	chicken (o/m)
1268	1275	✓	W						not in the UK, but reported from USA (wild bird), Canada (environmental waters), the Netherlands (environmental waters) and Sweden (wild bird)
1304		✓	W						not in the UK, but reported from Sweden (Wild bird)
1309		✓	E,W						not in the UK, but reported from Sweden (Wild bird) and Luxembourg (environmental waters)
1319		✓	E						not in the UK, but reported from Sweden (wild bird)
1540	1275				✓	E,F,W		gastroenteritis	Chicken.
1701	45				✓	E		gastroenteritis	chicken (o/m)
2068	2068				✓	E		gastroenteritis	
2111	952	✓	E					gastroenteritis	
2195					✓	E		gastroenteritis	chicken (o/m), turkey (o/m)
2209	179			✓					wild bird
2216		✓	E,W						wild bird, chicken
2274		✓	E		✓	BS		gastroenteritis	chicken
2538		✓	E						not in the UK, but reported from New Zealand (wild bird)
2655		✓	E,W					gastroenteritis	
2678		✓	W						wild bird

Cont.

2761	952	✓	E,W		✓	W			cattle
2793	1034	✓	W					gastroenteritis	chicken
2875		✓	W						not in the UK, but reported from Canada (human gastroenteritis, duck) and the USA (human gastroenteritis)
3149					✓	W		gastroenteritis	
3322		✓	W						duck
3502		✓	W						not in the UK, but reported from Sweden (chicken) and Luxembourg (environmental water)
3534		✓	E					gastroenteritis	duck
3704					✓	E,W			other animal
3925	1275	✓	W					gastroenteritis	
4028	952	✓	E		✓	E			not in the UK, but reported from Canada (environmental water)
4382		✓	E						not in the UK, but reported from Canada (wild bird)
4447	179	✓	W	✓					chicken
4569	952	✓	E					gastroenteritis	
4792		✓	E						not in the UK, but reported from Denmark (chicken o/m)
4843		✓	E						sheep
4879	1287	✓	W						not in the UK, but reported from The Netherlands (environmental water)
5136	464	✓	F		✓	E,F,BS		gastroenteritis	chicken
5559		✓	W						not in the UK, but reported from Sweden (chicken) and Luxembourg (environmental water)
5673		✓	W						not in the UK, but reported from Finland (human blood culture)
5845					✓	E			not in the UK, but reported from Luxembourg (environmental water)
5846		✓	W						not in the UK, but reported from Luxembourg (environmental water)
5982		✓	E					gastroenteritis	

Cont.

5985		✓	E						not in the UK, but reported from Luxembourg (environmental water)
5990		✓	W						not in the UK, but reported from Luxembourg (environmental water)
6065		✓	W						not in the UK, but reported from Denmark (chicken)
6168		✓	W						not in the UK, but reported from Luxembourg (environmental water)
6228	952	✓	E,W		✓	E			not in the UK, but reported from Belgium (farm environment) and Lithuania (wild bird)
6306	952	✓	W						not in the UK, but reported from Luxembourg (environmental water)
6427		✓	E						not in the UK, but reported from Lithuania (wild bird)
6518					✓	E,BS,W			not in the UK, but reported from Finland (environmental water)
6550		✓	W						not in the UK, but reported from Sweden (wild bird)
6557		✓	W						chicken
6759	353	✓	F					gastroenteritis	chicken
6985	61	✓	E,F,W	✓					unknown source
Novel 1				✓					most similar to those belonging to ST-45 complex
Novel 2				✓					most similar to ST 436, 3401, 3402, 3412, 3413, 5321, 5593

E	ENVIRONMENTAL
F	FAECAL
BS	BOOTSOCK (INSIDE CHICKEN SHEDS)
W	WATER